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(57) Abstract

The present invention comprises a novel method for detecting the presence of a target molecule in a sample and determining its molecular weight and concentration. The method involves the determination of electrokinetic velocities by measuring the time required for individual molecules labeled with at least two fluorescent probes to travel a fixed distance between two laser beams. Comparison of the electrophoretic velocity known to be characteristic of particular molecular weight standards with that of the target species permits determination of the molecular weight of the target. Alternatively, a two probe single detection zone method utilizing mechanical, electroosmotic or electrokinetic flow of a sample through a transport tube permits the identification of a target molecule in a complex test sample. The method is extremely sensitive, allowing one to detect and determine in a matter of seconds the molecular weight of a target molecule present in a concentration as small as one femtomolar.

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METHOD AND APPARATUS FOR SINGLE MOLECULE TWO COLOR FLUORESCENT DETECTION AND MOLECULAR WEIGHT AND CONCENTRATION DETERMINATION

This invention was made with Government support under Contract (No. W-7405-ENG-36) awarded by the United States Department of Energy. The Government has certain rights in the invention.

Field of the Invention

This invention relates to methods for detecting specific nucleic acid sequences in biological samples with high sensitivity and selectivity. The invention methods also relate to determination of the molecular weight and concentration of the target molecule.

Summary of the Related Art

The rapid and efficient detection of specific nucleic acid sequences in biological samples plays a central role in a variety of fields, including molecular biology, biotechnology, immunology, medical diagnosis and forensic analysis. Current techniques for detecting specific nucleic acids, notably Southern blotting, suffer from a variety of difficulties, including lack of sensitivity, time-consuming procedures, and necessity of large amounts of target DNA in the analytical sample. The polymerase chain reaction (PCR) has partially addressed the sensitivity issuc, but PCR and related amplification techniques (ligase chain reaction) often introduce spurious products that can cloud the interpretation of experimental results. Moreover, amplification methods, such as inverse PCR, are difficult to perform (*e.g.*, length of restriction fragments generated is unpredictable, re-ligation of fragment into closed loops is problematic, and a restriction site is required within the known sequence)

when the DNA sequences to be amplified are outside the known sequence of the target fragment.

Southern, J. Mol. Biol. **98**: 503 (1975), describes a technique for isolating particular DNA sequences by digesting genomic DNA with one or more restriction enzymes, separating the fragments according to size by electrophoresis (usually through agarose gel), denaturing the DNA *in situ*, transferring the DNA to solid support (nylon membrane), and hybridizing a labeled (radioactive, fluorescent, or colorimetric) probe to the specific sequence of interest. The hybridized probe is visualized according to its label type, and the presence of the sequence of interest can be detected and localized to a restriction fragment of a particular length. This technique is time consuming, requires large quantities of genomic DNA (~ 10 μg), and lacks sensitivity when short probes are used.

McCord et al., J. Chromatography **652**: 75 (1993), describes capillary electrophoresis of polymerase-chain reaction amplified DNA using fluorescence detection with the intercalating dye YO-PRO-1. Primer specific amplification can be used to detect the presence of specific DNA sequences in genomic DNA. This method lacks sensitivity (the lower limit of detection is 500 pg/ml) and selectivity (spurious band formation due to non-specific primer binding) and requires the use of an additional intercalating dye (ethidium bromide) for molecular weight resolution of less than 6 base pairs.

Cummins et al., PCT Appl. No. WO 96/06189, describes the detection of oligonucleotides in quantitative fashion by capillary electrophoresis. The use of labeled peptide-nucleic acid (PNA) oligomers to hybridize to specific oligonucleotide sequences is demonstrated. The method suffers from limited detection sensitivity and

cannot detect single copy genes in large genomes.

Eigen and Rigler, Proc. Nat'l. Acad. Sci., USA 91: 5740 (1994), describes the use of fluorescence correlation spectroscopy (FCS) to determine the presence of a single target molecule in a test sample. In FCS the diffusion of molecules in solution excited to fluorescence are observed and correlated such that when the excitation and detection are carried out in extremely small, confocally defined volume elements (0.2 fl), fluctuations at the level of single molecules can be observed. In particular, the interaction of a fluorescent ligand (*e.g.*, labeled oligonucleotide) with a larger target (DNA template) can be measured by the correlation function describing the diffusion of the free and bound ligand. Instead of correlating the fluctuations of one signal, the fluctuations of two signals with different characteristics can be cross-correlated. Increased signal-to-noise can be obtained by cross-correlation of two fluctuating signals. FCS detection takes place in a small light cavity (0.2 fl) without the use of separation processes (electrophoresis, column chromatography, or filter separation). The FCS method is limited by the requirement that extremely small volumes with very dilute solutions be used. A small volume light cavity is required because the diffusion times of the target molecules within the light cavity must be kept short enough to prevent the bleaching of the fluorescent dye, and the fraction of target molecules simultaneously present in the light cavity must be limited to achieve detection of single molecules. Also, a small volume is necessary to reduce scatter noise, which otherwise would obscure single molecule detection in visible colors.

Keller, et al., Appl. Spectroscopy 50: 12A (1996), describes the usc of two tags, one attached to a probe molecule and one attached to a target molecule, to assay a sample for the presence of a target. The assay can be done at the single-molecule level if there is a signal associated with the bound probe/target complex that is not

present with either the unbound probe or the unbound target. In this fashion, prior separation of the unbound and bound probes is not required, however, in cases where there is a large excess of probe (*e.g.*, when detecting multiple targets), separation or avoidance of free probe is desirable to speed analysis. Moreover, Keller, et al. does not specify how to attach a probe molecule to the target, indicating that it is often difficult or inconvenient to do so.

Shera, U.S. Patent No. 5,209,834, describes a method and apparatus for maintenance of a uniform motion of particles in a transport tube. The method is accomplished by the use of a transport tube, such as a capillary, composed of or coated with a material that when in contact with an electrolyte an electric double layer is formed at the wall/electrolyte interface. Applying an electric potential difference between the ends of the transport tube will produce an electroosmotic flow in a desired direction in the tube, regardless of their transverse position. Under the action of the electric field the electrolyte moves parallel to the electrolyte/wall interface such that particles placed within the electrolyte are maintained in the same order in which they were introduced into the transport tube. Particles transported in this fashion will have a constant transit time substantially independent of their radial position in the tube. If the particles are charged, the field gradients introduce an electrophoretic velocity component onto the electroosmotic velocity. Using this mode of transport through a capillary tube, detection of fluorescently labeled nucleotides cleaved from a given DNA sequence can be accomplished. Based on the transit times, the identity of each single nucleotide can be ascertained, thus enabling one to determine the linear sequence of the given DNA.

Castro and Shera, Anal. Chem. **67**: 3181 (1995), describes the use of Single Molecule Electrophoresis (SME) for the identification of single molecules in solution.

The technique involves the determination of electrophoretic velocities by measuring the time required for individual molecules labeled with a single fluorophore to travel a fixed distance between two laser beams. The use of a 0.25% hydroxypropylmethyl cellulose solution in the capillary tube enables the determination of the molecular weight of different sized labeled molecules based on their relative migration rates from one laser light illuminated zone to another. This technique has been applied to intercalated dye containing DNA, fluorescent proteins and fluorophores. An advantage of SME over conventional capillary zone electrophoresis (CZE) is that SME is a continuous flow system that permits real-time analysis, which is important in cases where sample concentration and composition change with time. There is, however, a disadvantage of the system described by Castro and Shera when applied to the detection of a specific DNA sequence within a large genomic background. The use of a single fluorescent probe complementary to the sequence of interest can still bind non-specifically to other sequences in the genomic DNA, resulting in detection of a false positive. In addition, unbound probe will also produce detectable signal that could be misinterpreted as the presence of the sequence of interest.

Several different types of targets and probes have been used in the methods described above. Of particular interest is identification of genes or specific DNA sequences in genomic DNA. Traditional techniques hybridizing DNA or RNA probes to DNA targets can suffer from a lack of specificity or affinity especially under conditions in which hybridization is unfavored (low-salt) or formation of secondary structure by the target interferes with DNA-DNA or RNA-DNA binding. Orum, et al., BioTechniques 19: 472 (1995), demonstrates the use of peptide-nucleic acid (PNA) probes to bind a 37 nucleotide, stem-loop structure target DNA sequence in 2M urea and high (500 mM) and low salt conditions. This reference shows that a PNA can

hybridize to its complementary nucleic acid over a broad range of salt concentrations without loss of affinity or specificity.

In view of the aforementioned deficiencies in the prior art, an improved method of rapidly detecting target nucleic acids with high sensitivity and determining its molecular weight and concentration is desirable.

SUMMARY OF THE INVENTION

The present invention provides a new method for detecting the presence of a target molecule in a sample and determining its molecular weight and concentration. The method employs means by which single molecule fluorescent emission can be detected. Consequently, the method is extremely sensitive, allowing one to detect and determine in a matter of seconds the molecular weight of a target molecule present in a concentration as small as one femtomolar. In addition, this method can detect the presence of a target within a sample much more rapidly than previous techniques and without separation of sample components. This is a vast improvement over such standard techniques as Southern blotting and capillary zone electrophoresis, which involve much longer processing times and orders of magnitude larger target concentrations (often necessitating molecular amplification by PCR).

An illustrative embodiment of the invention generally comprises contacting a solution of target nucleic acid molecules with two fluorophore-bearing peptide-nucleic acid (PNA) probes for sufficient time to allow hybridization of the probes to the target molecule, thereby forming a target-probe conjugate. The two probes are chosen to be complementary to different sequences within the target nucleic acid so that they can specifically and simultaneously hybridize to the target. The two probes bear different fluorophores that emit at different wavelengths. The resultant solution generally

comprises the conjugate among an excess of unbound probe. The resultant solution is injected into a capillary where it is subject to a force which induces the flow of the sample components down the length of the capillary.

In one embodiment, as the conjugate and unbound probes travel down the capillary they pass through two detection zones a known, fixed distance apart. Each detection zone comprises a region of the capillary into which a laser beam is focused. As the fluorophore-bearing conjugates and unbound probes pass through the first detection zone, the fluorophores undergo several cycles of excitation and emission. Two detectors (one for each color of light emitted from the two fluorophores) are set to detect the emitted light. Simultaneous detection of both fluorophores identifies the emitting species as the conjugate since unbound probe, bearing a single fluorophore, will emit at a single frequency only. Similarly, two other detectors detect emission in the second detection zone. Once again, simultaneous emission (and detection) from both fluorophores identifies the emitting species as the conjugate.

Because the distance between the two detection zones is known, the velocity of the conjugates can be determined by dividing the distance between the detection zone by the time it takes for the conjugate to travel between the zones. By comparing this velocity to that of standards of known molecular weight, the molecular weight of the target nucleic acid can be determined. The standards are identified in the same way as the target — two fluorescently labeled probes specific for the standard are added to the solution containing both the target and standard. If the standard is an internal one, at least one of the fluorescent labels on the standard's probe emits at a different frequency than both of the fluorophores on the target's probes. Alternatively, the standard can have the same fluorescent labels as the target and can be distinguished from the target by electrophoretic velocity.

Because the method is capable of detecting individual molecules, the concentration of the molecules can also be determined by comparing the number of target molecules detected to th number of molecules of a standard having a known concentration. For example, if one wishes to determine the number of copies of a target nucleic acid that have been successfully transfected into a cell, one can compare the number of targets detected to the number of a known nucleic acid having a known number of copies within the cell. Alternatively, if one wishes to know the absolute concentration of a target nucleic acid in a suspension, one can spike the suspension with a known concentration of a standard.

In another embodiment the conjugate (target hybridized to two fluorescently labeled oligomer probes) is detected in a single detection zone. Electrokinetic, electroosmotic or mechanical means are used to move the test sample through the capillary and into the detection zone. The simultaneous presence of the fluorophores can be detected in the manner described above. This embodiment permits the detection of a target in a complex sample as well as the concentration of the target in the presence of a standard, but does not permit the determination of molecular weight based on the transit time of the target between two detection zones.

An advantageous feature of the present invention is that the simultaneous detection of two fluorophores obviates the need to separate the conjugates from the unbound probe. Indeed, the method requires no separation of molecules, which is another factor effecting the efficiency of the method.

The present method can be advantageously used for a variety of applications. Most notably, the method can be used to replace the more time consuming and cumbersome Southern Blot method. It may be used to detect the presence of single gene copy within a cell to verify successful transfection of the cell and confirm that

only a single copy of the gene has been transfected.

The method can be used for a number of forensic purposes as well. For instance, the method is capable of routinely detecting concentrations of nucleic acids down to the femtomolar range without introducing ambiguities or errors resulting from PCR. Lower concentrations can be tested utilizing increased analysis times.

The foregoing merely summarizes certain aspects of the invention and is not intended, nor should it be construed, as limiting the invention in any way. All patents and other publications are hereby incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of the double label coincidence analysis.

Figure 2 is a schematic representation of the experimental set-up for the detection apparatus used to detect the a double labeled target molecule.

Figure 3a and 3b are schematic diagrams of the sample container and capillary set up.

Figure 4 is a schematic diagram of the output from detector 1 and detector 2 in the detection of a single copy *Bacillus thuringiensis* toxin gene obtained in Example 5.

Figure 5 is a histogram of the experimental result for the detection of a single copy *Bacillus thuringiensis* toxin gene obtained in Example 5.

Figures 6a-d are schematic diagrams of how the detection of the presence of a conjugate in two zones can be translated into a histogram that will indicate the presence or absence of a target nucleic acid.

Figure 7 is a graph of the detection of a single target gene produced from a computer simulation as outlined in Example 9.

Figure 8 is a graph of the detection of two targets produced from a computer simulation as outlined in Example 9.

Figure 9 is a graph of the detection of four targets produced from a computer simulation as outlined in Example 9.

Figures 10a-c are graphs of the detection of 30 targets produced from a computer simulation as outlined in Example 11.

Figure 11 is a plot of the minimum signal-to-noisc ratio versus genome count which was derived from a computer simulation as outlined in Example 10.

Figure 12 is a plot detection zone height versus analysis time derived from a computer simulation as outlined in Example 12.

Figure 13 is a plot detection zone cross-section arca versus analysis time derived from a computer simulation as outlined in Example 12.

Figure 14 is a schematic representation of the movement of negatively charged conjugate and neutral or positively charged probe in an electric field.

Figure 15 is a histogram of the experimental result for the detection of a single copy λ genomic DNA in a no urea buffer obtained from Example 6.

Figures 16a and b are histograms of the experimental results for the detection of a near-infrared fluorophore obtained in Example 7 with and without time-gated detection, respectively.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides improved methods of detecting a target molecule of interest (*e.g.*, a nucleic acid) and optionally determining its molecular weight and concentration in solution. The methods generally comprise contacting a solution containing a target molecule of interest with two different fluorescently labeled probes that specifically and simultaneously bind to the target molecule to form a conjugate. As used herein, the term "conjugate" refers to a target molecule with two or more fluorophore-bearing probes attached. Each of the probes bears a different fluorophore that emits at a different wavelength. The probe-containing solution is inserted into a capillary and made to flow through the capillary. Depending on the purpose for which the technique is being conducted, one or more detection zones are situated along the length of the capillary. These detection zones comprise a locus in which a laser beam is focused and optics, and corresponding photon detection apparatus detect fluorescence emission of the fluorophores as they pass through the detection zone. As the fluorophore-bearing conjugates pass through the detection zone(s) they undergo repeated cycles of excitation and emission. The emitted photons are detected by time gated detectors (one for each wavelength of fluorescence) at each detection zone. Simultaneous emission from two or more fluorophores generally signals the presence of a conjugate in the detection zone. As will be described more fully below, depending on the desired result, the present methods can be used with a single detection zone or two or more detection zones.

In one aspect, the present invention comprises a method of detecting the presence of a target molecule. In this aspect, a sample containing the target molecule is contacted with a solution containing two fluorophore-bearing probes under conditions that allow for specific and simultaneous binding of the probes to the target.

The resultant solution is made to flow through a detection zone in a capillary via mechanical (*e.g.*, pump or plunger), electroosmotic, or electrokinetic means. Detection of simultaneous emission from both fluorophores, or non-radiative fluorescence energy transfer between the two fluorophores will generally signal the passage of a conjugate through the detection zone. (A description of non-radiative fluorescence energy transfer is found in Birks, "Photophysics of Aromatic Molecules", pp. 567-576, Wiley-Interscience (1970)). By making the solution sufficiently dilute, simultaneous passage of the two different unbound fluorophores through the detection zone can be reduced to a negligible level. Although only a single detection zone is required, multiple detection zones will increase the signal-to-noise ratio and improve detection sensitivity. This embodiment provides for the very rapid detection of a given target within a complex sample without separation of the sample components. In terms of sample preparation simplicity and detection rapidity, this method presents a significant improvement over the prior art.

In another embodiment of the invention the test sample is layered onto a capillary such that all of the target molecules are placed in the essentially the same location in the capillary. An electrical potential is applied and the target molecules move through the capillary to a single detection zone where the conjugates can be identified by the simultaneous emission of two fluorophores. (Karger and Frantisek Foret, in "Capillary Electrophoresis Technology" Marcel Dekker, Inc. New York).

In a second aspect, the present invention comprises a method of detecting the presence of a target molecule and determining its molecular weight. In this aspect a conjugate-containing solution is made to flow through a capillary by generating an electric potential along the length of the capillary. In this aspect, two or more detection zones are positioned along the length of the capillary. A combination of

electroosmotic and electrophoretic forces results in each molecular specie "i" (including both unbound probe and conjugate) traveling at its own velocity (v^i), depending on its particular charge, shapc, and size. As in the first aspect of the invcntion, the detection of simultaneous emission from two or more fluorophores in a detection zone signals the presence of a conjugate in the zone.

The molecular weight of the target molecule can be determined from the velocity of the conjugate. The velocity is measured by determining the time, Δt^i_{jk}, required for the emitting species "i" (conjugate or unbound probe) to travcl from detection zone "j"to detection zone "k," which are a known distance, d_{jk}, apart. As described more fully below, this is accomplished by measuring the time between simultaneous emissions of the conjugate in zone "j" and zone "k." The velocity of the conjugate is $v^i=d_{jk}/\Delta t^i_{jk}$. By measuring the migration velocity of several standards of known molecular weight, a standard curve of migration rates versus molecular weight can be constructed and the molecular weight of the conjugate thereby determined from its velocity.

Occasionally two different probes that are unbound to a target molecule or an unbound and a bound probe will pass through the detection zones simultaneously and result in a simultaneous emission and detection of the two fluorophores. Thesc false positive signals can be made negligibly small by choosing the experimental conditions wisely, for example by diluting the sample, .

As used herein, a "coherent signal" is observed when, after sufficient data has been collected, simultaneous emissions are observed predominantly in detection zone k a time Δt_{jk} after a simultaneous emission in zone j. As noted, random simultaneous emissions can occur, for example, when two unbound fluorophore-bearing probes

happen into the detection zone at the same time. If the probes have different velocities, such simultaneous emissions will not lead to coherent signals because of their random nature. Repeated simultaneous emissions at fixed times Δt_{jk} (which result in a coherent signal) will in general be generated predominantly by conjugates only. If the probes travel with the same velocity, they may produce a coherent signal, but the resulting velocity will generally be much larger (due to the smaller size of the probes) than the conjugate.

In either of the two foregoing aspects of the invention, the concentration of the target molecule can be determined. This is accomplished by using additional probes and fluorophores. To illustrate, in one embodiment four different probes A, B, C and D (with three or four different fluorophores) are chosen such that probes A and B simultaneously bind to the target, and probes C and D simultaneously bind to a standard of known concentration. The oligomer probes are labeled with fluorophore such that the pair A and B and the pair C and D each contains at least one fluorophore not contained in the other pair. Thus, each pair can be uniquely identified by a two-color emission. Three or four, depending on the number of fluorophores used, detectors are used in both detection zones to detect simultaneous emission from A and B and from C and D. Coherent simultaneous emission from A and B uniquely identifies the target, and coherent simultaneous emission from C and D uniquely identifies the standard. By comparing the number of simultaneous emissions of A and B a time Δt^{conj} apart to the number of simultaneous emissions of A and D a time Δt^{std} apart, one can determine the concentration of the target. Since the velocity of the conjugate and the standard generally will be different, both could be identified by probes A and B. If the conjugate's velocity is unknown, however, it is preferable if

the standard and conjugate are identified by a separate set of fluorophores, such that each contains at least one unique fluorophore. This same approach can be used with four or more unique fluorophore-bearing probes complementary to a target and two or more standards, provided that a method is available to identify each standard, either by its velocity (in which case several standards can share fluorophore combinations) or by the fact that each standard has at least one fluorophore not shared with any other standard.

The number of copies of a gene in a cell can be detected with the present method by lysing the cells and treating the lysate with fluorophore-bearing probes as described herein. In this embodiment an internal standard comprising an endogenous gene of known copy number is used as a standard.

Since the number of cells lysed in producing the test sample is unknown and the total number of hybridized molecules detected is less than 100% of the total number of hybridized molecules in the test sample, the absolute number of transfected gene copies is not known. Assuming that AB and CD are not disproportionately represented at the detector, however (*i.e.*, the relative number of AB and CD conjugates passing through the detector zone is representative of the relative number of those conjugates in the whole test sample) the ratio of AB:CD will reveal the copy number of the transfected host cells. For example, if the known copy number of the internal standard is 2 copies per genome and the ratio of AB:CD is 5:1, then the copy number of the transfected gene is 10 copies per genome.

In relatively rare instances where the target and standard share identical sequences three probes, A, B and C (all uniquely labeled) can be utilized in the previously described embodiments. One oligomer probe, having a unique fluorophore is complementary to the sequence of the target oligomer (B), one probe having a

second fluorophore is complementary to the internal standard (C), and one probe having a third fluorophore is complementary to both the target oligonucleotide and the internal standard (A). Thus, when a test sample containing both internal standard and target oligonucleotide is incubated with the three oligomer probes, two fully hybridized species should emerge: target oligonucleotide with oligomer probes A and B (AB) and internal standard with A and C (AC). AB and AC will share a common fluorophore, but will be distinguishable based on the simultaneous presence of the fluorophore on probe A and either the fluorophore on probe B or C. This approach can be applied to determine the genetic state of a target gene. By determining the number of nucleic acids relative to the internal standard in the progeny of a genetic cross, a determination can be made as to whether the progeny is heterozygous (contained on one chromosome) or homozygous (contained on both homologous chromosomes) for the target gene.

The use of multiple oligomer probes labeled with multiple fluorophores permits detection of multiple targets within a complex sample. The use of three fluorophore labels (X, Y, Z) permits the detection of three unique identifiable pairs (XY, XZ, YZ). If the pairs are coupled with targets of discretely different sizes, multiple targets can be uniquely identified by electrokinetic velocity coupled with two-color fluorescence emission (see Example 11 and Figures 10a-c). Four different fluorophores (V, X, Y, Z,) provide six unique pairs (VX, VY, VZ, XY, XZ,YZ).

These approaches can also be applied to measuring the level of gene expression manifested by the expression level of messenger RNA (mRNA). In this context the target nucleic acid is mRNA and the internal standard is a DNA of known sequence and copy number. The present invention permits the determination of the ratio of the target and standard such that the relative level of mRNA may be assessed.

Preparation of the test sample in this embodiment would require the presence of both the endogenous RNA and DNA of the host cell. Alternatively, the internal standard can be an RNA molecule if the relative expression level is to be measured.

The present invention can be used to detect restriction cleavage site polymorphisms by determining the presence or absence of a particular restriction endonuclease cleavage site. Two probes, one on either side of the restriction site within a target oligonucleotide, are added to a test sample after the test sample's treatment with the particular restriction enzyme. If the site is absent both probes will bind to the target oligonucleotide and be detected coincidentally. If, however the site is present, the target is cleaved and the probes will bind to separate fragment of the target, and simultaneous detection will not be observed. Alternatively, the presence of a restriction site distal to the two probes can be determined by measuring restriction fragment length. This method can also be used in reverse to determine if two fragments have been joined during a ligation reaction. Probes to two separate fragments can be designed. If the fragments are joined by ligase or by genetic recombination in a cell, the detection of the two probes will occur simultaneously. Unjoined fragments will bind to one probe but not the other. Therefore, no simultaneous detection of the probes would be observed.

The present invention can be used to examine the genomic context of a transgene as well as detect genetic transpositions. The detection of a genetic insertion, whether a transposon or a transgene, can be determined by hybridization of oligomer probes to the genomic target sequence subsequent to the incubation of the test sample with restriction endonucleases. The presence of a transgene or a transposon can be detected by the increase in molecular weight of the target restriction fragment in the test sample. If there is no change in the mass of the genomic target fragment, the

transgene or transposon has not inserted into this restriction fragment. If there is a decrease in mass of the fragment, the transposon has been removed from the site. Alternatively, the insertion or deletion of a transgene or transposon can be detected if one probe hybridizes to the genomic target sequence and the other probe hybridizes to the transposon. A coherent signal is obtained only when the transposon is inserted in the target sequence. Single-nucleotide polymorphism can be detected according to whether at least one of the two target probes has a perfect match or is mismatched (Dulay et al., Poster #P-1110-T presented at the HPLC 96 meeting in San Francisco, CA on June 18, 1996).

Multiplex detection of individual gene targets can be accomplished by the present invention. Much like the determination of the ratio of target oligonucleotide to internal standard, multiple oligomer probes to several different target nucleic acids permit the detection of multiple gene targets in the same test sample. Multiplex detection using degenerate or partially degenerate probes could be used to determine the level of genetic identity in genetically uncharacterized organisms. Degenerate probes can be designed to hybridize to the 3' and 5' ends of repeated sequences so as to detect an undetermined number of the repeated sequence, many copies of which are dispersed through out the genome (e.g., Alu sequences in the human genome). A complex "fingerprint" would result from the analysis.

The target molecules can be nucleic acids, nucleic acid fragments, and other nucleic acid-like species that have specific nucleobase sequences (herein referred to as "targets" or "target sequences" or "target nucleic acids"). These target sequences may also be contained within a longer polynucleotide molecule. In particular, the invention provides methods for the detection of a specific transfected gene sequence against a background of other olig nucleotides r like species such as th se found

within an endonuclease treated DNA chromosomal preparation.

In preferred embodiments of the invention a mixture of nucleic acids, nucleic acid fragments, natural oligonucleotides, synthetic oligonucleotides, synthetic oligonucleosides and mixtures of one or more of these species suspected of containing a particular nucleotide sequence (test sample) is mixed with two labeled probes, each probe complementary to a portion of the target sequence. The probes hybridize under appropriate conditions to that portion of the target sequence to which they are complementary. At least a portion of this hybridization mixture is loaded onto a single molecule electrophoresis unit such that the doubly-labeled hybridization product can be distinguished from singly labeled hybridization products or unbound probes as described above. A single target sequence can be monitored and femtomolar or lower concentrations of the target sequence within a test sample may be detected. If necessary, samples containing a high concentration of target nucleic acid can be diluted to a suitable concentration before hybridization to oligomer probes.

The nucleic acids used in the invention (both target and probe) may be comprised of DNA or RNA (or both) having any of the naturally occurring bases (A, T, G, C, and U), non-naturally occurring bases (*e.g.*, I), and modifications thereof. Similarly, the target nucleic acid can be modified in the sugar unit or internucleoside linkage. Illustrative modifications to the sugar include, but are not limited to, 2' substituents (*e.g.*, 2'-*O*-methyl) or substitution of the entire ribose unit itself with, for example, furanose. Similarly, the internucleoside linkages can be modified in any of a variety of ways, including, but not limited to, phosphorothioate, phosphorodithioate, alkyl- and aryl-phosphonate and phosphonothioate, carbamate, etc. The only limitations are that the target and probes be sufficiently long and of such chemical

make-up that the two fluorophore-bearing probes can specifically hybridize to the target under conditions suitable for electrokinetic or mechanically induced flow of the test sample through the capillary. In a preferred embodiment of the invention, the target nucleic acid is a naturally occurring DNA (*e.g.*, a gene or fragment thereof) or RNA (*e.g.*, mRNA) polymer.

Any nucleic acid containing-organism (viral, prokaryotic, eukaryotic or archaebacterial) can serve as a source of target nucleic acid. In a preferred embodiment, the target oligonucleotide is a gene or gene fragment that has been transfected into a host organism. In another preferred embodiment the host organism is a plant, and in a most preferred embodiment the plant is corn, *Zea mays* or soybean, *Glycine max*. In certain preferred embodiments the gene or gene fragment codes for insect, pathogen or herbicide resistance.

"Oligomer probes" as defined in the present invention are naturally occurring or synthetic oligomers that hybridize to target nucleic acids. Hybridization is the sequence specific bonding of two species based on the complementarity of their sequences. Such bonding normally takes place through hydrogen bonds between exo-cyclic functional groups and ring nitrogens of the heterocyclic nucleobases. Classic Watson-Crick base pairing between A-T, G-C and A-U bases and Hoogsteen and reverse Hoogsteen base pairing as well as recently recognized "unusual" base pairings (G:A, G:T, G:U) are encompassed in hybridization based on complementarity. In a preferred embodiment the oligomer probes hybridize to target nucleic acids to form duplexes, triplexes or higher order structures. In a most preferred embodiment the oligomer probes hybridize to denatured RNA or DNA molecules to form a duplex.

In certain preferred embodiments, the oligomer probes are substantially electrically neutral species, that do not contain the multiplicity f negative charges

associated with the phosphodiester backbone of native DNA or RNA. The most preferred oligomer probes are peptide nucleic acids (PNAs), which contain a polyamide backbone and generally hybridize to target nucleic acids more strongly than oligonucleotides. Representative PNA species are disclosed in Orum, et al., BioTechniques 19: 472 (1995). Particularly preferred PNAs are those that incorporate a positively charged amino acid, such as lysine.

Each oligomer probe has a fluorophore attached to it, preferably at the oligomer's 3' or 5' end. Essentially any relatively small fluorophore may be used. Preferred fluorophores are fluoroscein, dansyl, fluoroscamine, OPA, NDA, rhodamine 6G, JOE, FAM, Texas Red, Cy5, Cy7, IRD41 and Bodipy-TR.

Oligomer probes according to the present invention preferably have between 8 to 50 nucleobases, with 10 to 25 nucleobases preferred. Obviously, the length of the oligomer probe chosen will depend on the target nucleic acid sought as well as the conditions chosen to conduct hybridization of probe to target. It is a routine matter for one of skill in the art to choose the appropriate length oligomer probe suitable for the desired purpose.

As used herein, the term internal standard is a nucleic acid of known sequence that is endogenous to a host organism of interest at a known copy number. Oligomer probes can be hybridized to internal standards and can serve to determine the ratio of the number of target nucleic acids to internal standards. In a particular embodiment this ratio relates the relative number of endogenous sequence to transfected sequence to determine copy number of the transfected gene.

As used herein, the term external standard refers to nucleic acids of known molecular weight and/or concentration. If used for determining concentration, external standards are preferably target nucleic acids added to the test sample at a

known concentration. A single external standard may be used for the determination of the target nucleic acid concentration in the test sample. Several external standards all of known concentration could, however, be employed to more accurately determine the concentration of the target nucleic acid in the test sample by comparing the detected concentration of the standards versus the target. In another embodiment the external standards could be run separately from the sample providing similar conditions (buffer, salts, detection apparatus) were used.

The determination of the molecular weight of the target nucleic acid requires the use of one or more external standards that have different molecular weights. In a preferred embodiment, a plurality of external standards with molecular weights capable of being separated by capillary electrophoresis (*e.g.*, λ HindIII fragments, 123 bp DNA ladder, or 1 kb ladder, Life Technologies, Inc, Gaithersburg, MD) are included in the test sample and detected as described above to determine their electrophoretic velocity over a given distance for purposes of constructing a velocity v. molecular weight profile that can be used to determine the molecular weight of a nucleic acid of unknown weight. If the approximate size of the target nucleic acid is known two, external standards that have molecular weights that bracket that of the target may suffice for determining the molecular weight of the target. External standards can be run independently from the test sample provided that similar conditions are used. Preferably, however, they are all run together.

In a preferred embodiment in which the target nucleic acid is detected with the use of two oligomer probes, the output from two detectors appears as in Figure 4. Each vertical line (or peak) in Figure 4 represents the signal (*i.e.*, a burst of photons) from one or more fluorophores (A or B) in one of the zones as it passes through the

zone at a particular time. The simultaneous presence of a peak in line A and line B indicates the presence of the target oligonucleotide to which both oligomer probes are bound. Every fluorescent burst produced at the second zone by a given fluorophore will be delayed with respect to the burst produced at the first zone by a time equal to the interbeam distance, d_{jk}, divided by the electrokinetic velocity, v^i, of the conjugate. Thus, simultaneous peaks seen at zone k can also be seen at detector zone j, but delayed in time with respect to detector j by an amount $\Delta t_{jk}=d_{jk}/v^i$. The cross-correlation between detector j and detector k of these times (as shown in Figure 7) will produce a series of peaks, one for each molecular species present. In Figure 7, a single species is present, thus one peak is observed.

Data interpretation may be done by any convenient method. In one embodiment (time interval analysis), one measures the time (Δt_{jk}) between each simultaneous emission in zone j and each simultaneous emission in zone k for all $\Delta t_{jk} \leq \Delta t_{max}$, where Δt_{max} is an arbitrary time chosen to be greater than the suspected migration time of the conjugate $_t^{conj}$. As described below, however, additional detection zones offer additional benefits. A histogram is created in which the number of Δt_{jk} in the particular migration time range (*e.g.*, 0-5 msec, 5-10 msec, 10-15 msec, etc.) is displayed. When sufficient data is collected a peak will appear in the migration time range in which Δt^{conj} falls. It will be appreciated that a large migration time range results in a large number of Δt_{jk} that fall in that range, meaning fewer data points need be collected. One pays the price of accuracy, however. Conversely, a small histogram migration time range results in a more accurate determination of Δt^{conj}, but more data points need be collected to obtain a satisfactory signal-to-noise ratio (≥ 3) because fewer Δt_{jk} will fall in the smaller range. It is a routine matter for the

ordinary artisan to choose parameters suitable for the desired purpose.

The method is depicted schematically in Figures 6A-D. Figure 6A shows the output of detectors 1 and 2 in detection zone j and detectors 1′ and 2′ in detection zone k. Detectors 1 and 1′ detect the presence of a first fluorophore in zones j and k, respectively, and detectors 2 and 2′ detect the presence of a second fluorophore in zones j and k, respectively. The solid vertical lines shown along each of lines 1, 2, 1′ and 2′ represent the detection of a fluorescent emission signal by each of the corresponding detectors. The horizontal direction is a time axis. A signal from each of detectors 1 and 2 (or 1′ and 2′) at the same position along the horizontal axis represents a simultaneous emission and detection from the two fluorophores in detection zone j (or k). The numbers appearing below lines 2 and 2′ are the times at which simultaneous emission from both fluorophores occurred in each of the detection zones; the small letters serve to identify each simultaneous emission.

In order to correlate the simultaneous emissions in zone j with those in zone k, the time between a simultaneous emission in zone j and subsequent simultaneous emissions in zone k (Δt_{jk}) is determined. As noted previously, an arbitrary time, Δt_{max}, is chosen to be greater than the suspected migration time between the zones of the conjugate, Δt^{conj}. Of course, Δt^{conj} is usually not known, but Δt_{max} can be chosen arbitrarily large to ensure $\Delta t_{max} > \Delta t^{conj}$. Then, for each simultaneous emission in zone j, the time between it and each subsequent emission in zone k is determined for all $\Delta t_{jk} < \Delta t_{max}$. In this illustrative example, $\Delta t^{conj} \approx 2.2$ ms and $\Delta t_{max} = 7$ ms. The Δt_{jk} are tabulated in Fig. 6B. Each row is a simultaneous emission in zone j, and each column is a simultaneous emission in zone k. Each cell represents the time between those simultaneous emissions in each row and column in which the cell resides.

After the data have been tabulated, a histogram is created. A "bin" time width is chosen. In Fig. 6C the bin time width is 1 ms and in Fig. 6D it is 0.5 ms. Then the number of Δt_{jk} in a particular bin (*e.g.*, 0-1 ms, 1-2 ms, 2-3 ms, etc., for Fig. 6C, and 0-0.5, 0.5-1.0, 1.0-1.5, etc., for Fig. 6D) is counted from the table in Fig. 6B and plotted in the histogram. The results are shown in the histograms in Figs. 6C and 6D. A peak in the histogram is presumed to be indicative of a target when S/N $\geq$ 3. In a dilute sample the random elements are reduced and the noise decreases relative to the signal.

Another method of detecting the conjugate and determining $\_t^{conj}$ is to take the cross-correlation of the data set consisting of the intensities of the simultaneous emissions of the fluorophores in the first detection zone as a function of time, $D_j(t)$, with the intensities in the second detection zone, $D_k(t)$: $D_j(t) * D_k(t_j)$. The resulting spectrum will exhibit a peak at $\_t^{conj}$.

Other embodiments include a greater number of detection sites such that the target oligonucleotide can be detected at three or more spatially separated detection zones. The output from multiple detectors can be correlated as previously discussed using migration velocities between zones 1 and 2, 2 and 3, etc. Improved signal-to-noise ratio can be achieved in the same amount of time as when two detection zones are used because there will be 2, 3 or more data points ($\_t_{1,2}$, $\_t_{2,3}$ etc.) obtained.

In another embodiment the target nucleic acid can be detected by three or more oligomer probes which hybridize to different portions of the target and have different fluorescent labels. The simultaneous detection of the three wavelengths emitted by the three oligomer probes verifies the presence of a single nucleic acid target. Detection of the target hybridized to three probes can be accomplished at two or more laser detector zones as outlined above. Obviously the use of more than two oligomer

probes with more than two fluorescent labels requires the presence of additional photodiode groups or photodetector arrays to detect the fluorescent emission from the additional oligomer probes. The use of three or more probes increases the S/N ratio by reducing the likelihood of simultaneous emissions from all probes other than those bound to the target.

In all methods, in which the free probe is not physically separated from the target-probe hybrid, including those of the prior art and the present invention, detection becomes more problematic when the fluorophore probe is present in large excess as compared to the target. This is because multiple fluorophores, due to their high concentration, can be simultaneously present at a detector, and thus signal a false positive. Dilution of the sample reduces this problem, but increases the time required for analysis of the sample. The selection of probes of unequal molecular weight would obviate the emission of a coherent signal from the same probe pair at multiple detectors. It would be preferable, however, to prevent any free probe from traversing the path of the detection zone.

Cummins et al., PCT Appl. No. WO 96/06189, describes the use of lysine-PNA probes in the context of capillary zone electrophoresis as a method of preventing unbound probe from moving through the detection zone. The incorporation of lysine in the uncharged PNA molecule gives the PNA(lysine) a single positive charge such that when subjected to an electric field, the lysine-PNA will move toward the cathode. Conversely, the negatively charged DNA-PNA(lysine) will move in the opposite direction. The detection zones can be stationed such that unbound DNA-PNA(lysine) moves away from these zones and thus is never detected.

In another embodiment of the present invention, therefore, probes are selected to move in a direction opposite to that of the conjugate in an electric field. Selection

of the probes requires only that the probes have a significantly different charge-to-mass ratio than that of the target molecule. In the preferred embodiment the probes are PNAs with a neutral or near neutral charge and the target is a DNA molecule. A sample containing target molecules is incubated with probes under hybridizing conditions and the sample is loaded into a capillary tube. Detection zones are located at one end of the capillary tube. An electric field is applied along the capillary under conditions such that the conjugate and free target move in opposition to the electroosmotic flow (EO), (and thus, toward the end of the capillary with the detection zones), and the unbound probes move with the electroosmotic flow and thus away from the detection zones. EO can be regulated by capillary coatings or by altering the pH of the electrophoresis running buffer (*See*, Regnier and Wu, in "Capillary Derivatization of Fused Silica Capillaries", p. 288 (1993)). After an initial period of time, all of the free probe originally located in the region between the detectors and the end of the capillary will have moved out of the detection zones, due to the EO, and toward the opposite end of the capillary as demonstrated in Figure 14. Once free probes are removed from the detection zones, detection of the conjugate may commence as previously described. Although some conjugates will have already cleared the detection zones, most will still remain in the portion of the capillary above the detection zones and provide sufficient signal for analysis.

In another embodiment, unbound probe initially present at the detection zones is eliminated by photobleaching or by backflushing the area of the detection zones with electrophoresis buffer before application of the electric field. Photobleaching can be accomplished by focusing a laser beam axially inside a capillary that has a 90 degree bend near the detectors. (*See*, Affleck, et al., Anal. Chem. **68**: 2270 (1996)).

The following represents a particular embodiment of the invention. It will be appreciated by those skilled in the art that many of the parameters and apparatuses are not critical features of the invention, and, therefore, variations can be made without exceeding the spirit or the scope of the invention. The artisan is referred in particular to Castro and Shera, Appl. Optics **34**: 3218 (1995) and Castro and Shera, Anal. Chem **67**: 3181 (1995) for their teachings regarding experimental parameters and apparatuses, all of which are suitable for use in the methods of the present invention.

In the present invention a test sample is heated such that the nucleic acids within the sample are denatured. The sample is then mixed with an excess of two PNA probes labeled with two different fluorescent labels. If any molecules of the target oligonucleotide are present, both probes will hybridize to the target to form a conjugate. Conditions can be selected by art recognized methods to induce sequence specific hybridization (*See*, Young and Anderson, in "Nucleic Acid Hybidisation: A Practical Approach", IRL Press, Oxford). The solution is incubated at a temperature that is above the melting temperature, T_m, of the probe-target hybrid. The temperature is lowered to just below the lower of the two probes' T_m. In this way random, non-specific binding is minimized. After incubation of three hours or less the test sample is loaded into the apparatus similar to that shown in Figures 3a and 3b. In an illustrative embodiment, the ends of a 2 cm long 100 x 100 μm cross-section capillary are connected to two sample reservoirs by means of tubing. Preferably, the sample volume required to fill the sample compartment is ~3 ml, but accommodation of smaller sample volumes can be accomplished by reducing the scale of the sample reservoir apparatus to reduce the volume to as little as 1 μl. The sample compartment can contain water or various electrophoretic media typically used in capillary

electrophoresis. Such materials include, but are not limited to: hydroxyethyl cellulose, hydroxymethyl cellulose, hydroxypropyl cellulose, uncrosslinked polyacrylamide and polyethylene oxide (*See*, Kuhn and Hoffstetter-Kuhn in "Capillary Electrophoresis: Principle and Practice", pp. 315-319 (1993)).

In a preferred embodiment the capillary is made of glass, but other substances transparent to light in the range of 200-1000 nm or higher, such as quartz, fused silica, and organic materials such as teflon, nylon, polyvinylchloride, polystyrene and polyethylene are appropriate. In a preferred embodiment the capillary is square, but other geometric configurations, such as rectangular or cylindrical, can be used also. A high-voltage power supply is connected to the sample reservoirs by means of platinum electrodes, for example. After the sample containing the target nucleic acid is placed in the sample reservoir a voltage is applied to induce electrophoretic migration through the detection zones. In a preferred embodiment 200 V/cm are applied, but other voltages in the range 10-1000 V/cm are appropriate.

Figure 2 shows a general schematic diagram for the detection apparatus of the present invention. Multiple lasers can, of course, be used, but preferably only a single laser is used as the excitation source. In some cases, all fluorophores can be excited simultaneously by the same laser light source. Certain fluorophores do not have a large enough Stokes shift, however, making it necessary to use multiple light sources emitting different wavelengths to excite different fluorophores. The laser is attenuated by a variable neutral density filter and then split into two parallel beams by a beam splitter-mirror combination. The resulting 1-5 mW laser beams are focused by a focusing lens into the sample capillary cell by an achromatic lens to yield two detection zones (defined by the region within the capillary in which the focused laser beam is of sufficient intensity to excite the fluorophores) separated by a given

distance. In a preferred embodiment the zones are about 10 μm in diameter and separated on the capillary by a distance of about 250 μm as shown in Figure 3a. Longer separation distances improve resolution. In conjunction with the selection of certain fluorophores, the laser is pulsed and the detectors gated off during each pulse. This eliminates detection of Rayleigh and Raman scattering, which occur within the duration of the laser pulse. The detectors are gated on between the pulses to detect fluorescence emission, which generally has a longer half-life than Raylcigh and Raman scattering. The lascr pulsc ratc should be set by considering, *inter alia*, the intensity of the laser, the fluorescence half-life of the fluorophores, and photobleaching effects. A suitable and typical pulse rate is about 80 MHz. Those skilled in the art will appreciate that selection of the pulse pattern and the laser wavelength depend on the particular fluorophores being used.

Selection of fluorophores with large Stokes shifts or which emit at wavelengths in the infrared region of the electromagnetic spectrum may obviate the need for time gated detection. For example, the use of IRD41 (Li-Cor, Lincoln, NE) as a fluorophore in the detection method dcscribcd above does not require time gated detection for unambiguous determination of its presence. In both cases, however, the laser wavelength must be selected that will excite the fluorophore to fluoresce at a level that will be detectable by the optics and electronics of the system.

As a conjugate passes through the detection zone, thc fluorophorcs of the probes absorb the incident light, which causes them to enter an excited electronic state. As they relax from this excited statc to the ground electronic state the fluorophores emit a characteristic wavelength. There will be two separate cmission intensities and wavelengths corresponding to the two separate fluorophores hybridized

to the target nucleic acid.

Repeated excitation-emission cycles produce a fluorescence photon burst that is collected by a microscope objective and spatially filtered by a slit that defines a detection area for each laser beam. The two fluorescent emissions, one from each laser detection zone, are then separated by a mirror that deflects one beam relative to the other. The zones must be large enough to enable a sufficient number of conjugates to pass through and be detected in both zones. Although the method can be used successfully if the zones are less than the full width of the capillary, some conjugates will miss one or both zones, thereby reducing the sampling efficiency of the method. Preferably, therefore, the zones comprise the full width of the capillary so that each conjugate will necessarily pass through both zones.

The length of the zone (*i.e.*, the dimension in the direction of flow) should be adjusted to maximize sensitivity of the method. The longer the zone, the more excitation and emission cycles each fluorophore undergoes. But by increasing the zone length, one also increases the likelihood that two unbound fluorophores will simultaneously pass through the zone and emit, leading to false positive signals. False positive signals will also arise when the sample is too concentrated, since the more concentrated the solution the greater the likelihood that two unbound fluorophores will simultaneously pass through the detection zones. It is a routine matter for the skilled artisan to adjust the laser intensity, the size of the detection zone and the concentration (*e.g.*, by diluting it) to obtain a suitable (if not maximal) signal-to-noise ratio. Each fluorescent emission is then spectrally filtered by a bandpass interference filter, optimized to discriminate among fluorophores, and detected by photodiodes. Suitable parameters and apparatuses include a 40x, 0.75 NA microscope objective, a

0.4 x 0.4 mm square slit that defines a 10 x 10 μm detection area for each laser beam, and a photodetector that is a E.G.& G single photon avalanche photodiode. It is also possible to image both detection zones with one objective lens and one set of detectors. It will be recognized that alternate microscope objectives, slit geometries and widths, and detectors can be used and are within the spirit of the present invention. It will be appreciated that the particular optics are not critical features of the invention and any combination of optical elements, including optical fibers that allow simultaneous detection of one or more detection zones, can be used.

Each detector output signal is analyzed by independent time correlated single-photon-counting electronics under computer control. The time correlated single photon counting technique allows the determination of the arrival time of single photons to the detector, measured with respect to the arrival time of the laser pulse to the sample (*See e.g.* O'Connor and Phillips, *Time Correlated Single Photon Counting*, Academic Press (1984)). The detection electronics reject Raman and Rayleigh scattering by using a time-gated window set such that only delayed fluorescence photons are detected, thus increasing the signal-to-noise ratio of single-molecule detection. In a preferred embodiment fluorescence data are collected at 1 ms intervals. The ability to distinguish photon bursts from each fluorophore depends not only on the concentration but the bin size, which is the time period during which photons emitted by the fluorophore are counted. It is a routine matter for the artisan to adjust these parameters to yield a suitable (if not maximal) signal-to-noise ratio. Typical bin sizes are 0.10 to 0.05 of the fluorescent burst width. The fluorescence burst width is the collection of photons emitted by a single molecule as it traverses a laser beam. The burst shape is a convolution between the molecular velocity function (usually linear)

and the laser intensity profile (usually a gaussian function). The width is measured at half the amplitude of the burst (i.e. FHWM). The burst FWHM is typically about 10 to 20 msec.

The following examples are offered for illustrative purposes only and are not intended, nor should they be construed, to limit the invention in any way. Those skilled in the art will appreciate that variation can be made without violating the spirit or scope of the invention.

EXAMPLES

Example 1

Sample Preparation

The tissue from a maize plant, transfected with a single copy of a *Bacillus thuringiensis* toxin (BT) gene per haploid genome was harvested and lyophilized for two days. After grinding the tissue in a 50 ml tube by shaking with ten 5/32" ball bearings in a paint shaker (Red Devil 5400, Model 6400-002) for five minutes, 1.6 g of the resultant ground tissue was placed in a separate 50 ml tube. 27 ml of CTAB Extraction Buffer (200 ml of 1M TrisCl, pH=7.5; 20 g of hexadecyltrimethylammonium bromide (CTAB); 81.76 g of NaCl; 40 ml of 0.5 M sodium ethyl (EDTA), pH=8.0; diluted with water to a final volume of 2 L) was added to the ground tissue, after which 270 µl of 14.3 M 2-mercaptoethanol was added. The mixture was heated for one hour in a 65°C water bath. After heating, 15 ml of chloroform:isoamyl alcohol (24:1) was added and the mixture shaken for five minutes. The tube was centrifuged for 20 minutes at 1100 x g and about 15 ml of supernatant recovered by aspiration with a pipet. The supernatant was placed in a second 50 ml tube and 30 ml of Precipitation Buffer (100 ml of 1M Tris-Cl, pH=7.5; 20 g of CTAB; 40 ml of NaEDTA, pH=8.0 diluted with water to a final volume of 2 L) was added. The contents of the tube were mixed by inversion, the tube was let stand at room temperature for 30 minutes and centrifuged for 10 minutes at 1100 x g. The supernatant was removed and the pellet was resuspended in 10 ml of 100 mM Tris-Cl, pH=7.5, 10 mM EDTA and 700 mM NaCl. 25 ml of ethanol was added by gently overlaying it on the aqueous phase. The DNA precipitated at the ethanol-aqueous interphase and was visible as a white stringy mass. The DNA was collected by

twirling a glass rod around it and it was then dried on a Kimwipe paper tissue. The DNA was dissolved in 5 ml of 10 mM Tris- Cl, pH=7.5 and 0.1 mM NaEDTA, and half the volume was transferred to a 13 x 100 mm glass tube. 125 µl of 10% sodium dodecyl sulfate (SDS) was added, and then 25 µl of 18 mg/ml Proteinase K (Boehringer-Mannheim, GmBH, (Indianapolis, IN) was added. The solution was incubated for 20 hours at 50°C. The solution was dialyzed against four 4 L changes (once every ten hours) of 10 mM Tris-Cl, pH=7.5, 0.1 mM NaEDTA and 0.5% SDS using 50,000 mw cut-off dialysis tubing. The solution was then dialyzed against four 4 L changes (once every ten hours) of 10 mM Tris-Cl, pH=7.5, 0.1 mM NaEDTA using 50,000 mw dialysis tubing. 350 µl of 3M NaCl and 8 ml of ethanol were added the dialysate to precipitate the DNA. The DNA was collected with a glass stirring rod and dried with a Kimwipe. The DNA was dissolved in 200 µl of a 0.1 mM NaEDTA solution and its concentration estimated by optical absorbance at 260 nm. The concentration of the DNA was approximately 5 µg/µl.

EXAMPLE 2

Peptide-Nucleic Acid Probe Preparation

The following PNA probes were purchased from PerSeptive Biosystems, Inc. (Framingham, MA):

SEQ ID NO. 1: 5'-GGA TGC ACT CGT TGA-Lys-Rho-3'

SEQ ID NO. 2: 5'-GCC AGT GTT GTA CCA-Lys-BodipyTR-3'

Lys is the amino acid lysine; Rho (rhodamine 6G), and BodipyTR (Bodipy Texas Red) are both fluorescent probes purchased from Molecular Probes, Inc. (Eugene, OR). A Probe Mix of Seq ID No. 1 and Seq. ID No. 2 was made by diluting 1µl of each probe (which had been dissolved in 10mM Tris-Cl and 0.1 mM NaEDTA to a

concentration of 50 μM) in 2.5 mL water so that the final concentration of each probe was 20 nM. The Probe Mix was vortexed and placed in -20°C freezer until use.

EXAMPLE 3

Hybridization Protocol

89 μL of TE-urea buffer (10 mM Tris-Cl, pH=7.5, 0.1 mM NaEDTA and 7 M urea) and 10 μl DNA sample (5 μg/ml) were added to a 1.5 ml polypropylene microfuge tube and incubated in a sand heating block for 4 minutes at 95°C so that the DNA sample was denatured. The tube was removed from the heating block and cooled at room temperature for at least 5 minutes. 0.5 μl of the Probe Mix (20 nM with respect to each probe) was added to the DNA sample and the solution was mixed by stirring with a pipet tip. The tube was placed in the dark at room temperature for 16-24 hours and the solution then frozen at -20°C until such time as it was to be analyzed.

EXAMPLE 4

Instrument Configuration

A Spectra-Physics 3800 frequency doubled mode-locked Nd:YAG laser producing 70 ps pulses at 532 nm wavelength and 82 MHZ repetition rate was used as the laser source in the set-up outlined in Figure 2. The laser was attenuated by a variable neutral density filter and then split into two parallel beams by a beam splitter-mirror combination. The resulting 1-5 mW laser beams were focused into the sample capillary cell by an achromatic lens to yield two 10 μm spots. The ends of the 2 cm long 100 x 100 μm square cross-section glass capillary were connected to two sample reservoirs (5 ml) by the means of plastic tubing (ID = 400μm and OD = 1000μm) The

total sample volume required to fill the sample compartment was ~3 ml. The output of a high-voltage power supply was connected to the sample reservoirs by means of platinum electrodes. The applied voltage was between 100-200 V/cm. As the individual target molecules moved through each laser beam due to the electrophoretic effect, repeated excitation-emission cycles produced a fluorescence photon burst that was collected by a 40x, 0.75 NA microscope objective and spatially filtered by a 0.4 x 0.4 mm square slit that defined a 10 x 10 μm detection area for each zone. The light was then spectrally filtered by 30 nm bandwidth, eight cavity interference filters centered at the wavelength emission maxima and detected by two E.G. & G. single photon avalanche photodiodes. Each detector output signal was analyzed by independent time correlated single-photon-counting electronics under computer control. The detection electronics reject Raman and Rayleigh scattering by using a time-gated window set such that only delayed fluorescence photons are detected, thus increasing the signal-to-noise ratio of single-molecule detection. Fluorescence data were collected at 1 ms intervals.

EXAMPLE 5

Detection of Single Gene Copy of Bacillus Thuringiensis Toxin in Transfected Maize

DNA was extracted from both a wild type corn plant and a transgenic corn plant containing one copy of a *Bacillus thuringiensis* toxin (BT) gene per haploid genome in the manner described above (Example 1). The extracted DNA (2.5×10^{-13} M in genome equivalents) was incubated at 95°C for four minutes. A four hundred-fold excess of probes SEQ ID1 and SEQ ID 2 (1×10^{-10} M) were incubated with the DNA sample in 10 mM Tris-Cl, pH=7.5; 1mM EDTA and 6.3 M urea for 16 hr at 21°C. After the incubation, 50 μl of sample diluted in 5 ml of water was loaded into

the detection apparatus and the diluted sample was pushed with a motorized syringe at 200 μm/sec. Fluorescence data were collected at 1 ms intervals. The BT target gene was identified by coincident detection of the two different fluorophore probes bound to the gene in a single detection zone. Figure 5 demonstrates the detection of the BT toxin gene in a single detection zone.

EXAMPLE 6

Detection of Phage λ DNA Target in Salmon Testes DNA

Background Utilizing Buffers with No Urea

Phage λ DNA (New England Biolabs, Beverly, MA) was mixed with salmon testes DNA (Sigma, St. Louis, MO) in a ratio of approximately one λ genome to one salmon genome. This DNA mixture (5 x 10^{-13} M in genome equivalents) was incubated in 300 μl of 10 mM Tris-Cl, 0.1 mM EDTA, pH=7.2 at 95°C for 4 minutes.

1.5 μl of a PNA probe mixture specific for λ DNA (20 nM each of SEQ ID 3 and SEQ ID 4, PerSeptive BioSystems, Framingham, MA) were added to the DNA mixture and incubated at 20°C for 12 hours.

SEQ ID NO. 3: 5'-TAT TTG ACG TGG TTT-Lys-Rho-3'

SEQ ID NO. 4: 5'-BodipyTR-O-GCC-TCC-ACG-CAC-GTT-$CONH_2$

After incubation, 50μl of sample, diluted in 5 ml of water was loaded into the detection apparatus and the diluted sample was pushed with a motorized syringe at 200 μm/sec. Fluorescence data were collected at 1 ms intervals. The λ target was identified by coincident detection of the two different fluorophore probes bound to the gene in a single detection zone. Figure 15 demonstrates the detection of the λ target in

a single detection zone.

EXAMPLE 7

Detection of a Near-InfraRed Fluorophore

IRD-41 (Li-Cor, Lincoln, NE) was diluted in methanol to a concentration of 200 fM. The sample was loaded onto the detection apparatus as described in Example 6. The excitation was accomplished with a 780 nm, 5.5 mW light source. Detection of fluorophore emission was accomplished with 815-850 nm bandpass filter and a photodetector. Figure 16 a and b demonstrate the detection of the target in a single detection zone with time-gating and without time-gating, respectively.

EXAMPLE 8

Computer Simulations for Multi-Fluorophore, Multivelocity Analysis

As previously described, the combination of two or more fluorophore probes in combination with the use of two or more detection zones can permit the detection of a target molecule by fluorophore emission and electrokinetic velocity. A computer model was used to characterize this system in the following manner.

The Monte Carlo algorithm consisted of 104857600 steps, each 5×10^{-6} sec in duration. During each step, the following events may or may not have occurred, consistent with their respective probabilities.

1) Generation of individual molecules at random positions and times at the top of the capillary cell. The time between molecules is an exponentially-distributed random number. The position and time generation is consistent with cell geometry and sample concentration.

2) Translation of molecules due to electrophoretic-electroosmotic migration and random diffusion. The simulation of electrophoretic-electroosmotic migration consisted of adding a constant velocity to each generated molecule in the direction of

the capillary axis. The translation due to diffusion follows a random walk model.

3) Excitation of molecules at their current positions. The probability of excitation depends on the number and position of the laser beams, their gaussian cross-section energy distribution, their focused energy distribution, their intensity, and the molecular absorption.

4) Poisson-distributed generation of fluorescence photons from each excited molecule in the capillary cell, which considered fluorescence quantum yield of the fluorophores.

5) Detection of molecular fluorescence, which considered detection geometry, photon collection efficiency, and random detection of noise (detector dark noise, unfiltered Raman light, unfiltered Rayleigh scattering and from fluorescence impurities).

6) Removal of photodestructed molecules, consistent with their photodestruction efficiency.

At the end of the simulation, the data consisted of the number of photons generated during each step at each detection channel. The data is then binned in 1msec bins and written to disk. A cross-correlation across the various detection channels data sets was then performed.

The following simulation parameters were used:

Parameter	Setting
Concentration	2.6×10^{14} molar
Diffusion coefficient	3.0×10^{-10} cm^2/sec

Absorption cross-section	$2.2 \times 10^{8}\ \mu m^{2}$
Fluorescence quantum yield	0.8
Photodestruction quantum efficiency	8.0×10^{-6}
Square capillary side dimension	20 μm
Number of laser beams	2
Distance between beams	250 μm
Total photon detection efficiency	5.0×10^{-3}
Laser wavelength	532 nm
Photon energy	3.8×10^{-19} joules
Laser pulses per second	8.2×10^{7}
Laser beam waist at $1/e^{2}$ intensity	5 μm
Laser power	10 mW
Total noise count rate	150 sec^{-1}

EXAMPLE 9

Computer Simulations of the Effect of Velocity and Fluorophore Combinations on Detection of Multiple Targets

The model sample contained from one to four targets distinguished by fluorophore combinations and migration velocities.

TARGET	VELOCITY (μm/s)	FLUOROPHORES
1	100	AB
2	800	AB
3	110	AC
4	120	AC

Three fluorophores (A, B, C) and two detection zones were utilized. The two detection zones were separated by 250 μm along the capillary.

In the first experiment, a single target, 1, was detected. This target had a velocity of 100 μm/s and thus required 2500 ms to traverse the 250 μm distance between detection zones, as shown by the peak at 2500 ms in figure 7. A signal-to-noise ratio of 100 was obtained in this case.

In the second experiment, the detection of two targets, one that migrates rapidly and one that migrates slowly, was explored. Target 1 and target 2, both of equal concentration and identified by the same fluorophore combination, were placed in the model sample. Again, a two detection zone scheme was utilized. As a consequence of target 2's eight-fold greater migration rate, eight times more of this target is seen by the detector than of target 1 during a given time element. As can be seen in Figure 8, the slow target (target 1) is somewhat obscured in the background due to the high count of the faster moving target 2. For a peak to be unambiguously detected, it must be at least three standard deviations above the mean background fluctuation (Long and Winefordner, Chemistry 55: 712A (1983)). That is, the signal-to-noise ratio must be greater than 3. In this simulation, a signal-to-noise ratio of 30

was obtained. This means that even when the velocity of one target is as high as 8 times that of another, the slower moving target can easily be detected, although with a reduced signal-to-noise ratio (*i.e.*, 100 for the first experiment versus 30 in this case).

In a final experiment, the impact of different fluorophore combinations on detection of the presence of a slow target in a background of fast target was ascertained. The conditions of the second experiment were repeated except that two additional slow targets, 3 and 4, were added to the model sample. As can be seen in Figure 9, targets 3 and 4 are not obscured by the presence of fast moving target 2. This indicates that it is possible to simultaneously detect target of different fluorophore pairs without interference.

EXAMPLE 10

Computer Simulation for the Determination of the Signal-to-Noise Ratio of Three Detection Zones v. Two Detection Zones

The model (a macro in Microsoft Excel 7.0) employed a random number generation program to position detection events randomly in time at a first detector. Correlated events were positioned at different times at other detectors according to the electrophoretic velocity of the subject target molecule and the distance between the detectors along the capillary. This routine generated a simulated data set of randomly dispersed events correlated in time across detectors. In concentrated samples, it was not possible to identify which events were correlated and which were not, because several individual target molecules may pass the first detector at random times before any of them pass the second detector. Time interval analysis was used to detect correlated events dispersed in a background of uncorrelated ones. For each event at the first detector, the time intervals to each neighboring event at the second detector

were recorded. Neighboring events in the second detector are those which occur within a specified time window relative to the subject event at the first detector. Correlated events of a particular target accumulate at a characteristic time interval, while uncorrelated events distribute uniformly across the time window. The results are plotted as a histogram where the peak height above the background of a correlated event is a direct count of the number of respective target molecules detected by the analysis. An alternative method of analysis is cross-correlation. The simulations described below were analyzed by thc time interval method.

In a system having two detectors, background noise arises from the inability to exclude uncorrelated events from the data set. Three detectors arrayed along thc capillary can identify some of the uncorrelated events that could bc excluded from the data set and consequently improve the signal-to-noisc ratio. For example, in a system of three equally spaced detectors, a particular event occurring at some time interval across detectors 1 and 2 will be considered only if it also occurs at twice that interval across detectors 1 and 3. To compare the signal-to-noise ratio of two and three detector systems, the thirty targets of Example 11 were analyzed using six different counts, ranging from 5 to 200 of the slowest moving targets. Peaks were identified only if they exceeded a signal-to-noise ratio of 3. In Figure 11 the minimum signal-to-noise ratio for a given set of identified peaks is plotted as a function of the target number present in the detected sample. Each data point is an average of six trials. The results demonstrate that a three detector system improves the relative signal-to-noise ratio compared to a two detector system by 1.4 to 3.1 over a range of 5 to 200 targets. In other terms, the three detector system reproducibly identified 100% of the target in 65 seconds (target count 20), whereas the two detector system required 160 seconds (target count 50) to identify 100% of the targets.

EXAMPLE 11

Computer Simulation of Multiplex Detection of Thirty Targets Distinguished by Velocity and Fluorophore Combinations

The model sample contained the thirty targets listed in the table below:

TARGET	VELOCITY (μm/s)	FLUOROPHORES
1	300	AB
2	330	AB
3	363	AB
4	399	AB
5	439	AB
6	483	AB
7	531	AB
8	585	AB
9	643	AB
10	707	AB
11	300	AC
12	330	AC
13	363	AC
14	399	AC

15	439	AC
16	483	AC
17	531	AC
18	585	AC
19	643	AC
20	707	AC
21	300	BC
22	330	BC
23	363	BC
24	399	BC
25	439	BC
26	483	BC
27	531	BC
28	585	BC
29	643	BC
30	707	BC

Two detection zones, 250 μm apart were utilized in the detection of the targets. A minimum of 50 of the slowest target passed the detection zones. Total analysis time

was 160 seconds. Assuming that each target was a single copy gene in a maize genome (3 x 10^9 base pairs per genome), the genomic DNA sample concentration was 1 ng/µl. Whereas all of the targets were detected in the same run, the results for each fluorophore combination were plotted separately, as can be seen in Figures 10a-c. All of the targets were reliably detected.

EXAMPLE 12

Computer Simulation of the Analysis Time as a Function of Detection Zone Height and Cross-Section Area

The detection zone volume is described by the shape of the focused laser beam in the capillary, in terms of its height along the capillary axis and the cross-sectional area perpendicular to this axis. To explore the effect of detection geometry on total analysis time, a model sample was created that contained 100 molecules of a target species moving at 400 µm/s. A significant constraint in these simulations was that the counting efficiency was always held constant as the detection geometry was varied. As the volume was increased the sample concentration was reduced according to Poisson probability in order to avoid a loss in efficiency due to the presence of multiple targets being present at the detector at the same time.

In the first experiment, to determine the influence of detection zone height on analysis time, the cross-sectional area was held at 4000 µm^2. As can be seen in Figure 12, the analysis time increased linearly with the detection zone height. As expected, the constraint on counting efficiency forced a reduction in sample concentration as the detection zone volume increased. The signal-to-noise ratio was constant at about 19 for all data points.

In the second experiment, to determine the influence of detection zone cross-

section area on analysis time, the cross-sectional area was varied between 400 and 4000 μm^2 and the height held constant at 2 μm. As can be seen in Figure 13, the analysis times was independent of the cross-section area of the detection zone. As expected, the constraint on counting efficiency forced a reduction in sample concentration as the detection zone volume increased. The signal-to-noise ratio was constant at about 19 for all data points.

The analysis time depends on the detection zone height, but is independent of detection zone cross-section area. The quickest analysis is achieved with a low-height laser beam and a high sample concentration.

Numerous modifications and variations in the invention as described in the above illustrative examples are expected to occur to those skilled in the art and consequently only such limitations as appear in the appended claims are to be placed thereon. Accordingly, it is intended in the appended claims to cover all such equivalent variations that come within the scope of the invention as claimed.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(I) APPLICANT: Castro, Alonso
Williams, John

(ii) TITLE OF INVENTION: Method and Apparatus for Single Molecule Two
Color Fluorescent Detection and Molecular Weight and Concentration
Determination

(iii) NUMBER OF SEQUENCES:4

(iv) CORRESPONDENCE ADDRESS:
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(C) CITY: Chicago
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(F) ZIP: 60606

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: Word Perfect 6.1

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME:GREENFIELD, MICHAEL S
(B) REGISTRATION NUMBER:37,142
(C) REFERENCE/DOCKET NUMBER:96,794

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE:312-715-1000
(B) TELEFAX:312-715-1234

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:15 base pairs
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single

(D) TOPOLOGY:linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGATCGACTC GTTGA
15

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCCAGTGTTG TACCA
15

(4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TATTTGACGT GGTTT
15

(5) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:15 base pairs
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCCTCCACGC ACGTT
15

We Claim:

1. A method of detecting a target nucleic acid in a sample comprising:

(a) simultaneously or sequentially adding to the sample under conditions suitable for hybridization at least two fluorophore bearing probes having sequences of nucleosidic bases that are complementary to at least a portion of the sequence of the target nucleic acid, wherein the probes are capable of simultaneous and specific hybridization to the target nucleic acid;

(b) placing at least a portion of thc probe-containing sample into a capillary having one or more detection zones;

(c) causing thc probe-containing sample to move through the capillary; and

(d) detecting the simultaneous emission of the fluorophore bearing probes at one or more detection zones.

2. The method of claim 1, wherein the fluorophore is selected from the group consisting of fluoroscein, dansyl, fluorescamine, OPA, NDA, rhodamine 6G, acridine, JOE, FAM, Bodipy-TR, Cy-5, Cy-7, and IRD41 provided that the first and second fluorophore containing probes contain different fluorophores.

3. The method of claim 1, wherein the number of detection zones is one.

4. The method of claim 1, wherein the number of detection zones is two or more.

5. The method of claim 1, wherein the fluorophore bearing probes are between 8-50 nucleobases in length.

6. The method of claim 1, wherein the fluorophore bearing probes are 10-25 nucleobases in length.

7. The method of claim 1, wherein the fluorophore bearing probes are selected from the group consisting of PNA, DNA, RNA and PNA-DNA hybrid molecules.

8. The method of claim 1, wherein the fluorophore bearing probes are PNA molecules having a charged amino acid at the 3' or 5' terminus.

9. The method of claim 8, wherein the charged amino acid is lysine or histidine.

10. The method of claim 1, wherein the sample is moved through the capillary electrokinetically or mechanically.

11. A method for determining the genomic copy number of a target nucleic acid in a sample containing an internal standard comprising:

(a) simultaneously or sequentially adding to the sample under conditions suitable for hybridization at least three fluorophore bearing probes having sequences of nucleosidic bases at least two of which are complementary to at least a portion of the sequence of the target nucleic acid and at least two of which are complementary to at least a portion of the sequence of the internal standard provided that no two probes are complementary to sequences in both the target and internal standard;

(b) placing at least a portion of the probe containing sample into a

capillary having one or more detection zones;

(c) causing the probe containing sample to move through the capillary;

(d) detecting the simultaneous emission of the fluorophore bearing probes at one or more detection zones; and

(e) comparing the number of simultaneous emissions of all the fluorophores on the target nucleic acid bound probes to the number of simultaneous emissions of all the fluorophores on the internal standard bound probes.

12. The method of claim 12, wherein the fluorophore is selected from the group consisting of fluoroscein, dansyl, fluorescamine, OPA, NDA, rhodamine 6G, acridine, JOE, FAM, Bodipy-TR, Cy-5, Cy-7, and Ird41 provided that the first and second fluorophore containing probes contain different fluorophores.

13. The method of claim 11, wherein the number of detection zones is one.

14. The method of claim 11, wherein the number of detection zones is two or more.

15. The method of claim 11, wherein the fluorophore bearing probes are between 8-50 nucleobases in length.

16. The method of claim 11, wherein the fluorophore bearing probes are 10-25 nucleobases in length.

17. The method of claim 11, wherein the fluorophore bearing probes are selected from the group consisting of PNA, DNA, RNA and PNA-DNA hybrid molecules.

18. The method of claim 17, wherein the flurophorc bearing probcs arc PNA molecules having a charged amino acid at the 3' or 5' terminus.

19. The method of claim 18, wherein the charged amino acid is lysine or histidine.

20. The method of claim 11, wherein the sample is moved through the capillary electrokinetically or mechnically.

21. A method for determining the molecular weight of a target nucleic acid in a sample containing a plurality of external or internal standards of known molecular weight comprising:

(a) simultaneously or sequentially adding to the sample under conditions suitable for hybridization at least three fluorophorc bearing probes having sequences of nucleosidic bases at least two of which are complementary to at least a portion of the sequence of the target nucleic acid and at lcast two of which arc complementary to at least a portion of the sequences of the external standards provided that no two probes are complementary to sequences in both the target and external standards or internal standards;

(b) placing at least a portion of the probe containing sample into a capillary having two or more detection zones;

(c) causing the probe containing sample to move electrokinetically through the capillary;

(d) detecting the simultaneous emission of the fluorophore bearing probes at two or more detection zones;

(e) determining the transit time of the target nucleic acid and the transit times of the external standards or internal standards between at least two detection zones; and

(f) comparing the transit time of the target nucleic acid to the transit times of the external standards or internal standards.

22. The method of claim 21, wherein the fluorophore is selected from the group consisting of fluoroscein, dansyl, fluorescamine, OPA, NDA, rhodamine 6G, acridine, JOE, FAM, Bodipy-TR, Cy-5, Cy-7, and IRD41 provided that the first and second fluorophore containing probes contain different fluorophores.

23. The method of claim 21, wherein the number of detection zones is one.

24. The method of claim 21, wherein the number of detection zones is two or more.

25. The method of claim 21, wherein the fluorophore bearing probes are between 8-50 nucleobases in length.

26. The method of claim 21, wherein the fluorophore bearing probes are 10-25 nucleobases in length.

27. The method of claim 21, wherein the fluorophore bearing probes are selected from the group consisting of PNA, DNA, RNA and PNA-DNA hybrid molecules.

28. The method of claim 27, wherein the flurophore bearing probes are PNA molecules having a charged amino acid at the 3' or 5' terminus.

29. The method of claim 21, wherein the charged amino acid is lysine or histidine.

30. The method of claim 21, wherein the sample is moved through the capillary electrokinetically or mechanically.

31. A method for determining the concentration of a target nucleic acid in a sample containing an external standard comprising:

(a) simultaneously or sequentially adding to the sample under conditions suitable for hybridization at least three fluorophore bearing probes having sequences of nucleosidic bases at least two of which are complementary to at least a portion of the sequence of the target nucleic acid and at least two of which are complementary to at least a portion of the sequences of the external standard provided that no two probes are complementary to sequences in both the target and external standard;

(b) placing at least a portion of the probe containing sample into a capillary having one or more detection zones;

(c) causing the probe containing sample to move electrokinetically or mechanically through the capillary; and

(d) detecting the simultaneous emission of the fluorophore bearing probes at one or more detection zones;

32. The method of claim 31, wherein the fluorophore is selected from the group consisting of fluorescein, dansyl, fluorescamine, OPA, NDA, rhodamine 6G, acridine, JOE, FAM, Bodipy-TR, Cy-5, Cy-7, and IRD41 provided that the first and

second fluorophore containing probes contain different fluorophores.

33. The method of claim 31, wherein the number of detection zones is one.

34. The method of claim 31, wherein the number of detection zones is two or more.

35. The method of claim 31, wherein the fluorophore bearing probes are between 8-50 nucleobases in length.

36. The method of claim 31, wherein the fluorophore bearing probes are 10-25 nucleobases in length.

37. The method of claim 31, wherein the fluorophore bearing probes are selected from the group consisting of PNA, DNA, RNA and PNA-DNA hybrid molecules.

38. The method of claim 37, wherein the flurophore bearing probes are PNA molecules having a charged amino acid at the 3' or 5' terminus.

39. The method of claim 31, wherein the charged amino acid is lysine or histidine.

40. The method of claim 31, wherein the sample is moved through the capillary electrokinetically or mechanically.

FIG. 1

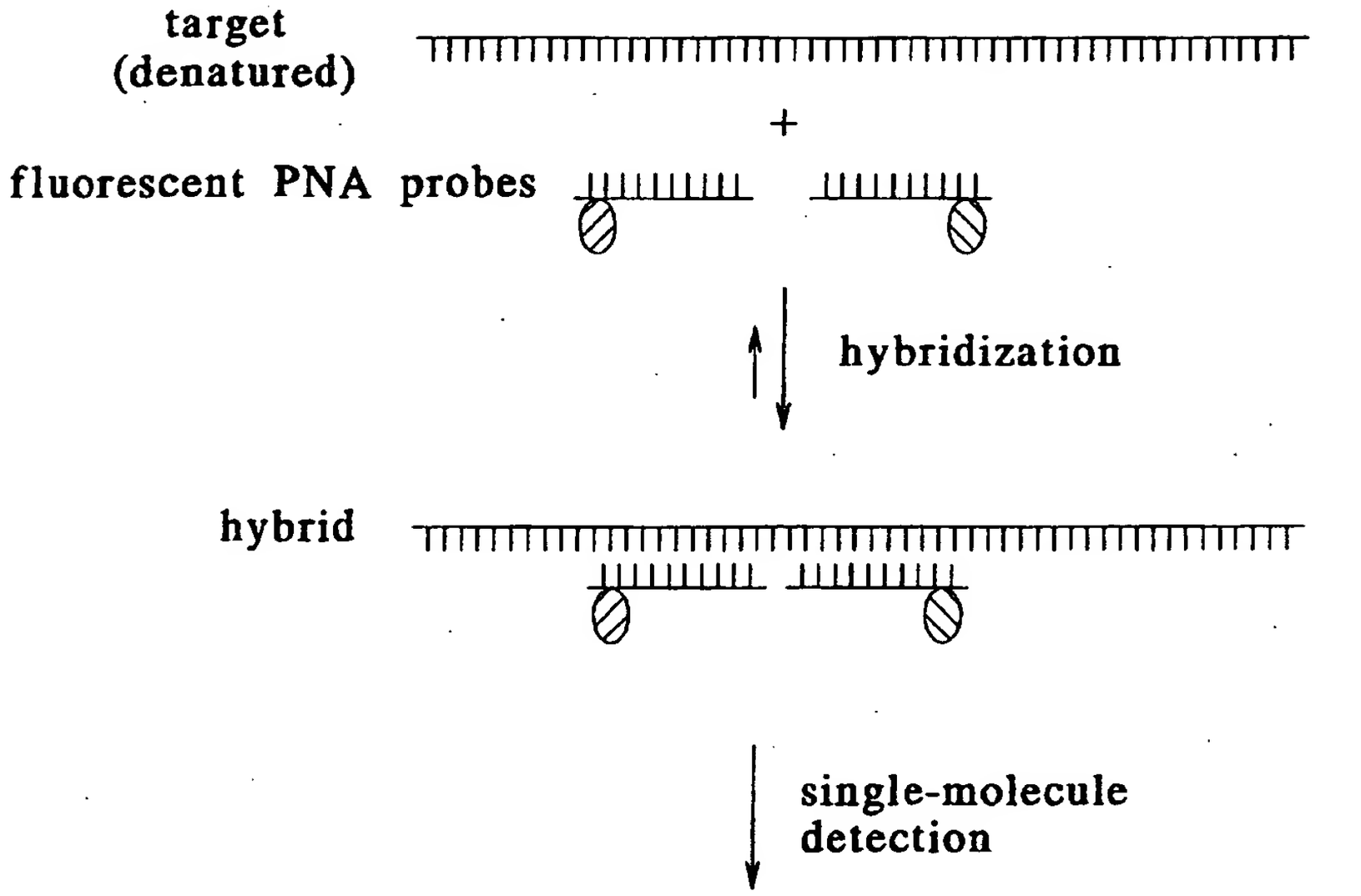

target
(denatured)
+
fluorescent PNA probes
hybridization
hybrid
single-molecule
detection
green channel
red channel
target

FIG. 2

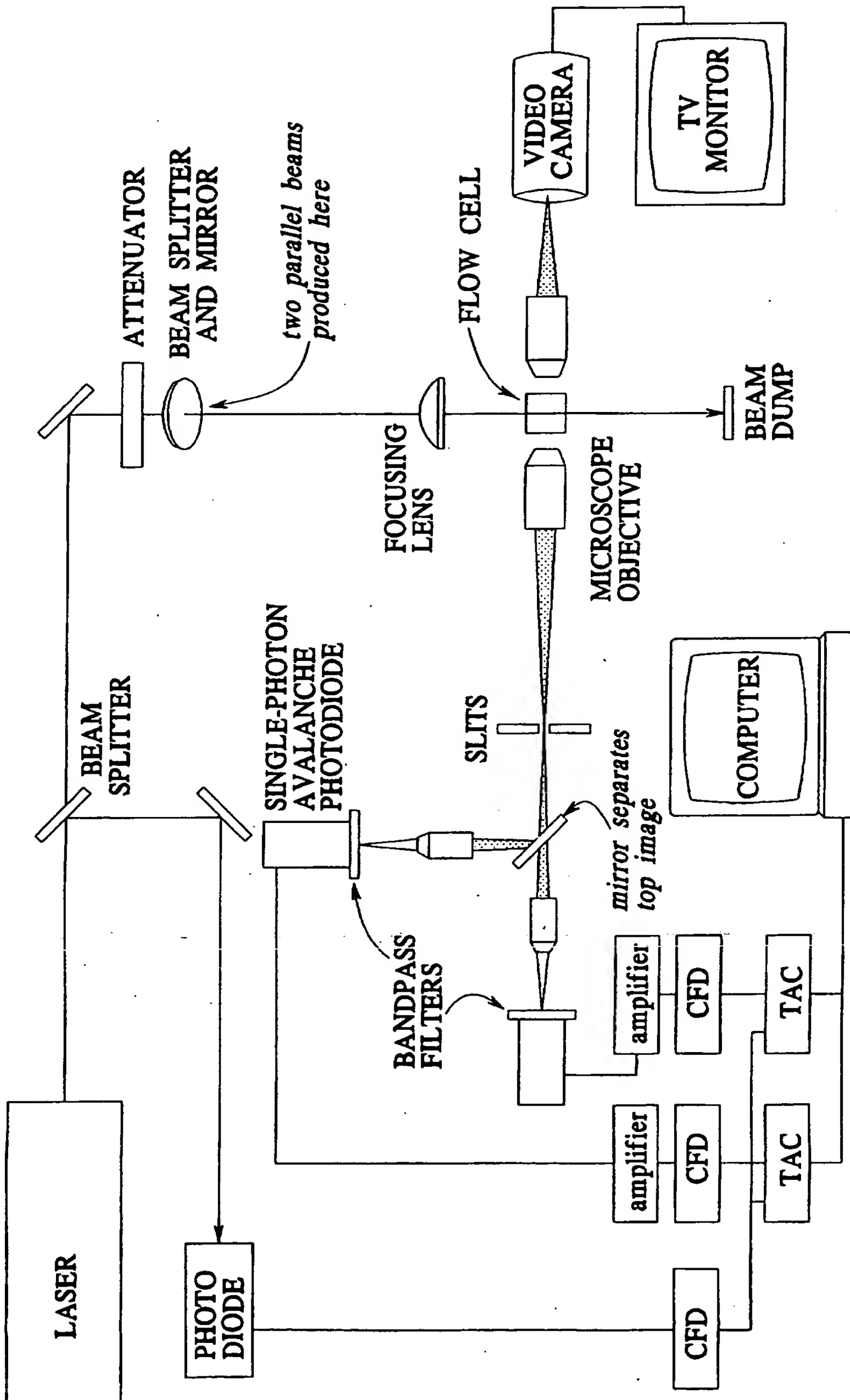

ATTENUATOR
BEAM SPLITTER AND MIRROR
two parallel beams produced here
FLOW CELL
VIDEO CAMERA
TV MONITOR
BEAM DUMP
FOCUSING LENS
MICROSCOPE OBJECTIVE
BEAM SPLITTER
SINGLE-PHOTON AVALANCHE PHOTODIODE
SLITS
COMPUTER
mirror separates top image
BANDPASS FILTERS
amplifier
CFD
TAC
amplifier
CFD
TAC
CFD
LASER
PHOTO DIODE

FIG. 3A

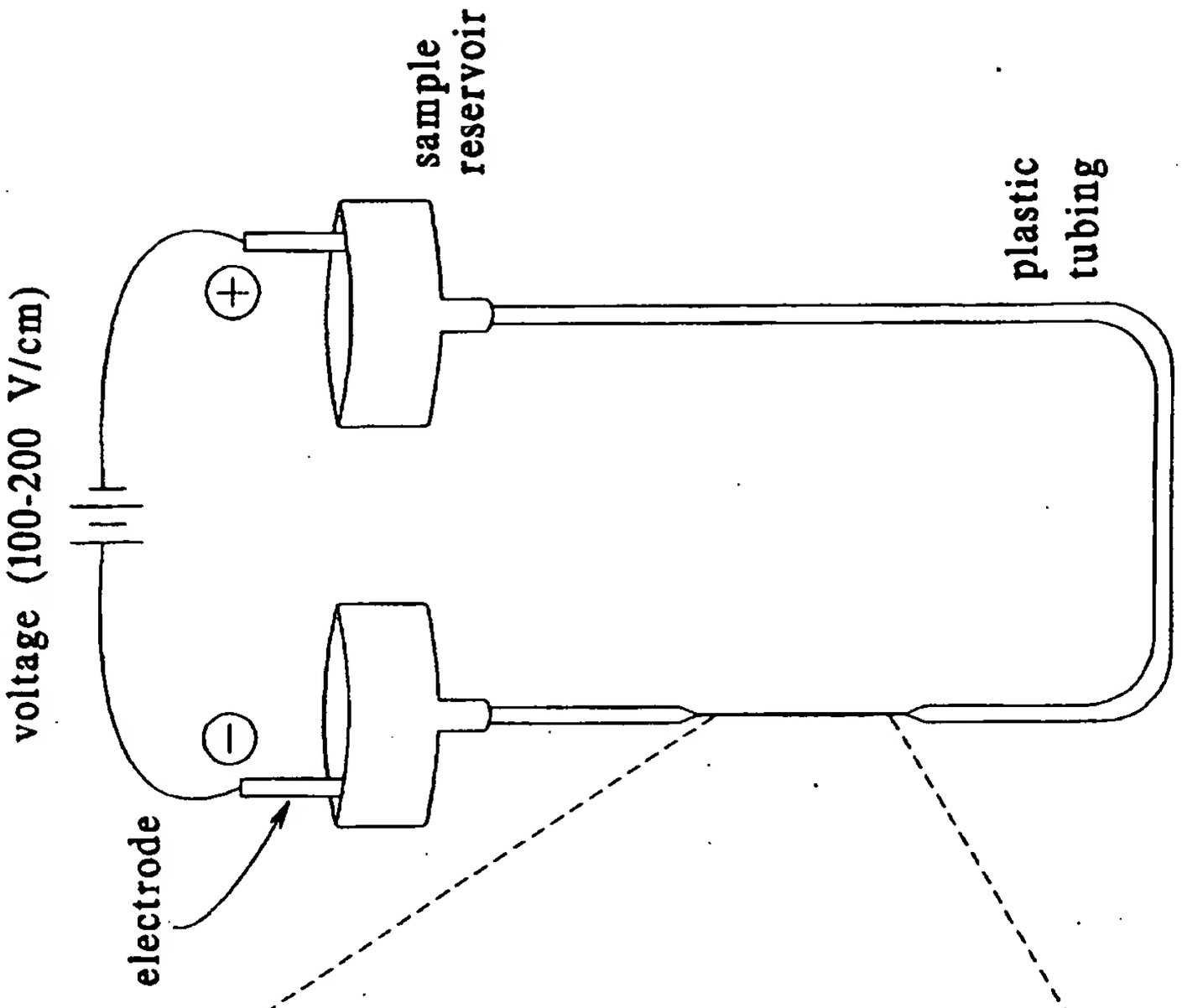

sample reservoir
plastic tubing
voltage (100-200 V/cm)
electrode

FIG. 3B

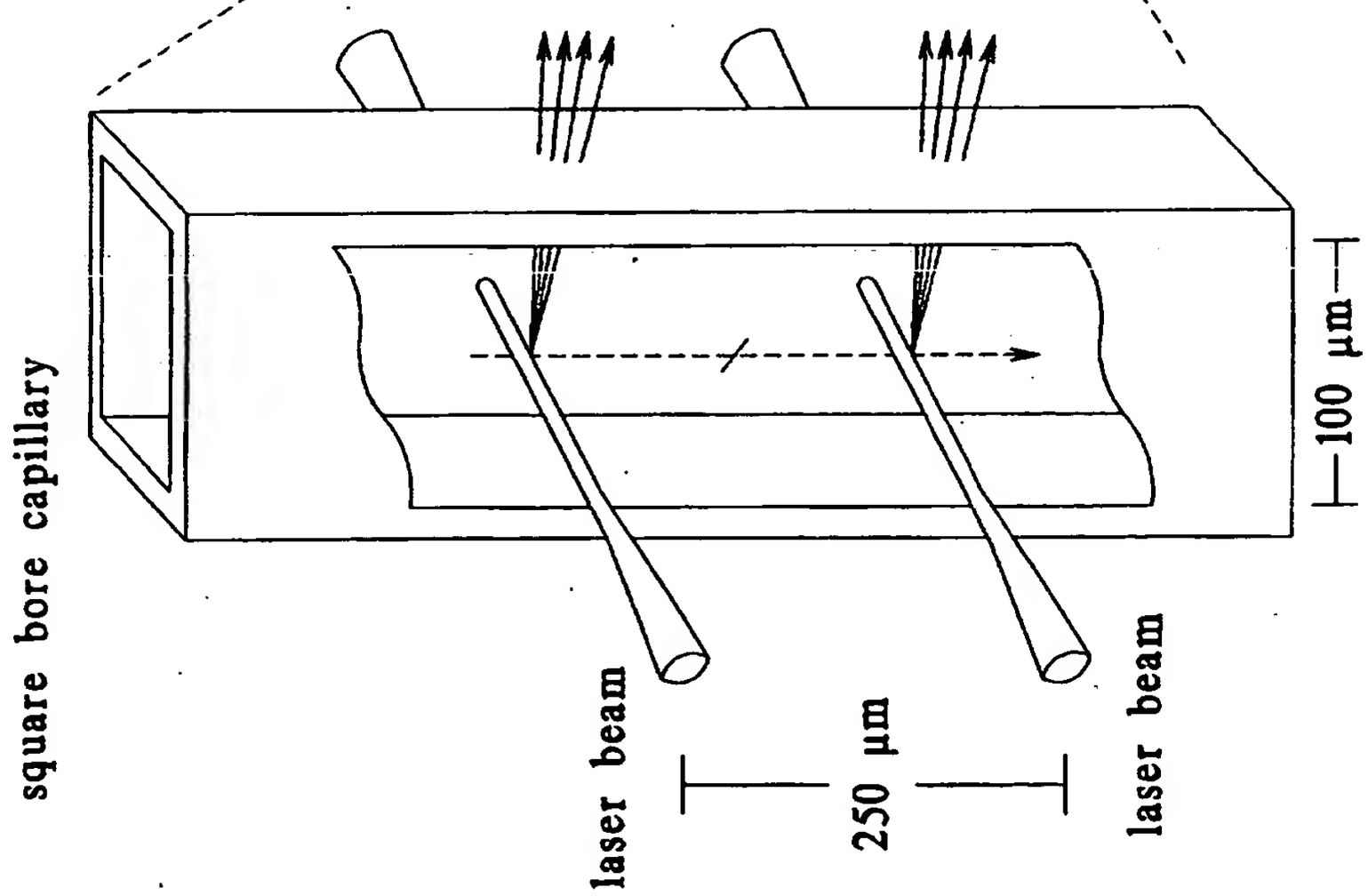
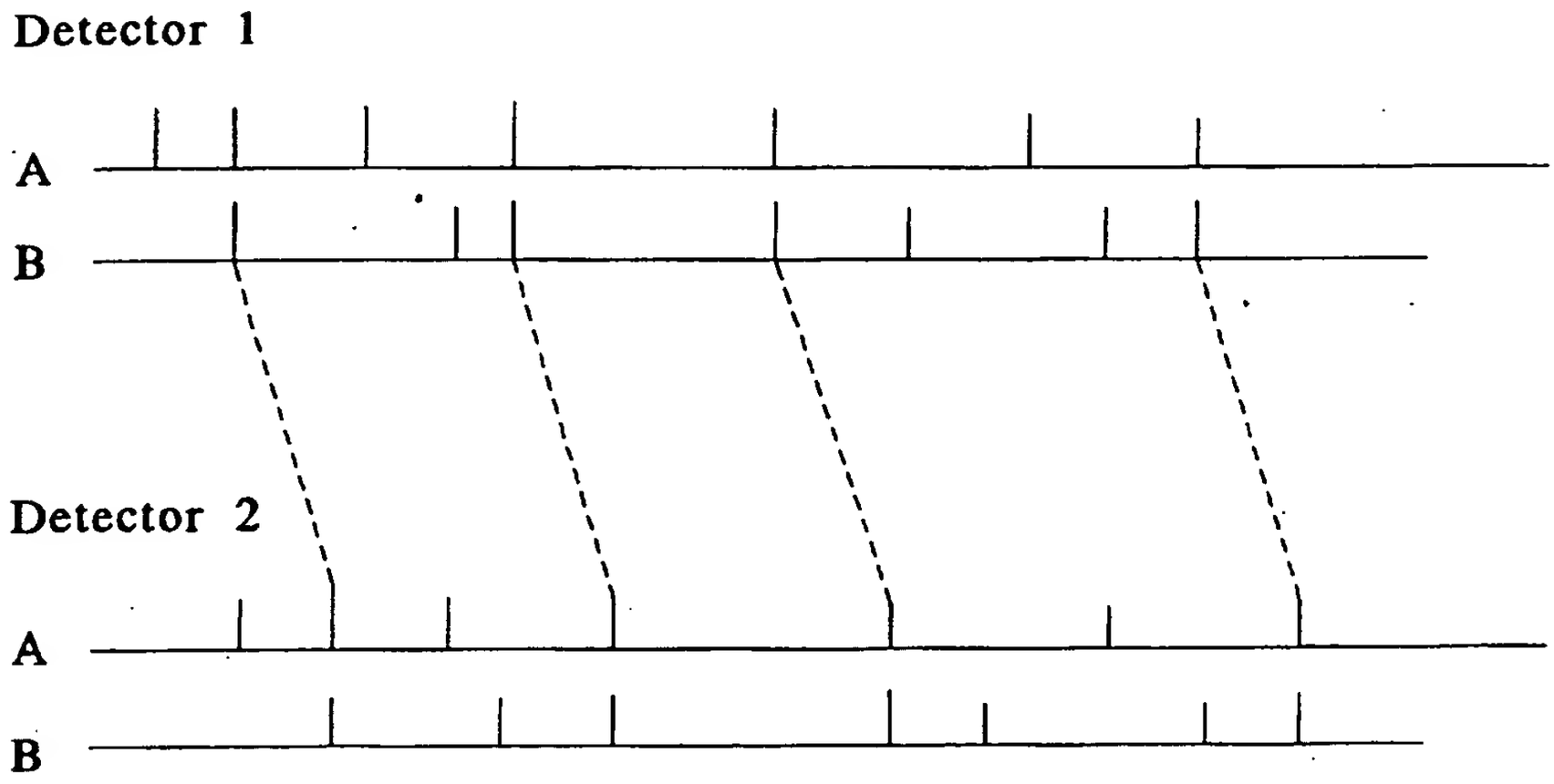

square bore capillary
100 µm
laser beam
250 µm
laser beam

FIG. 4



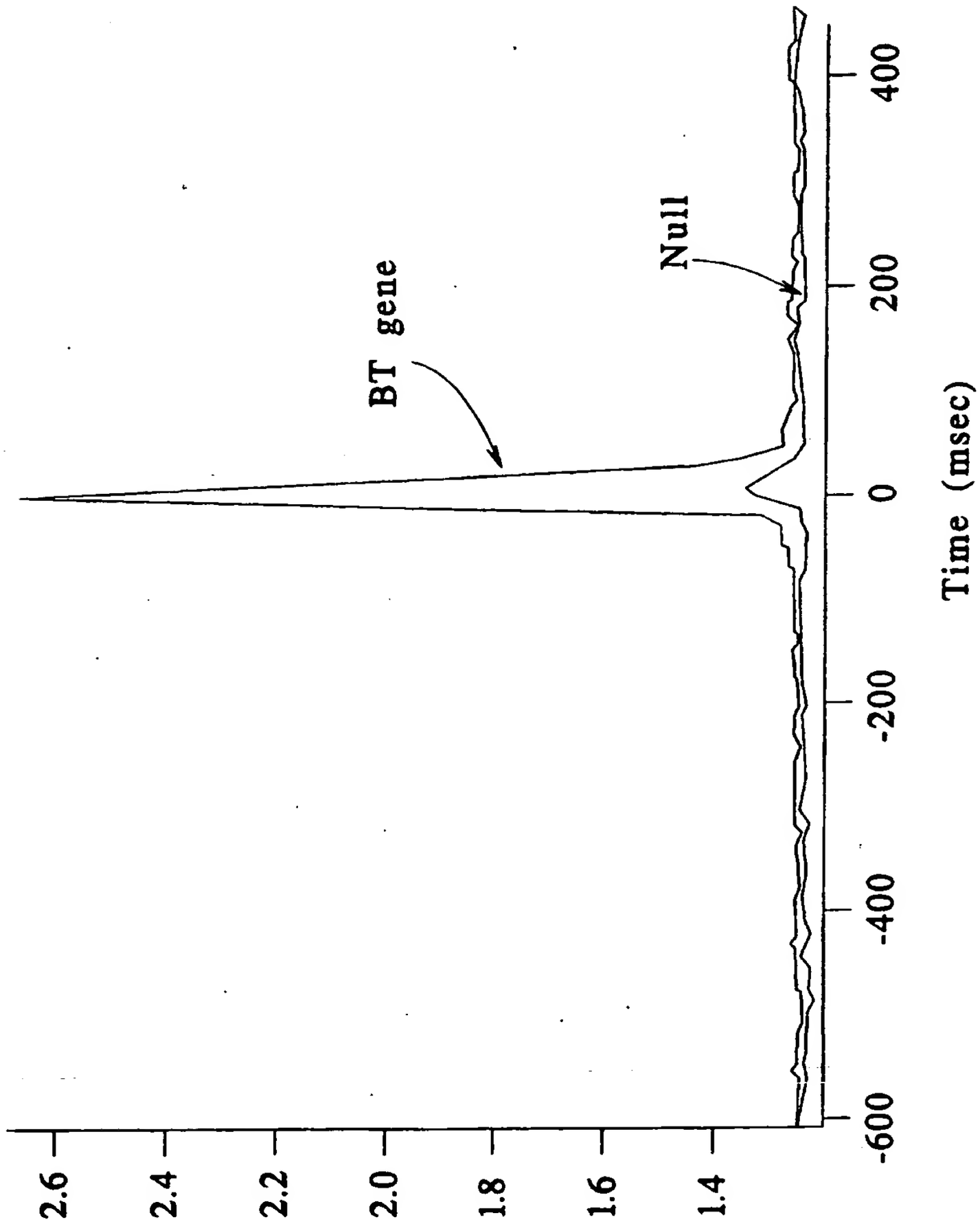
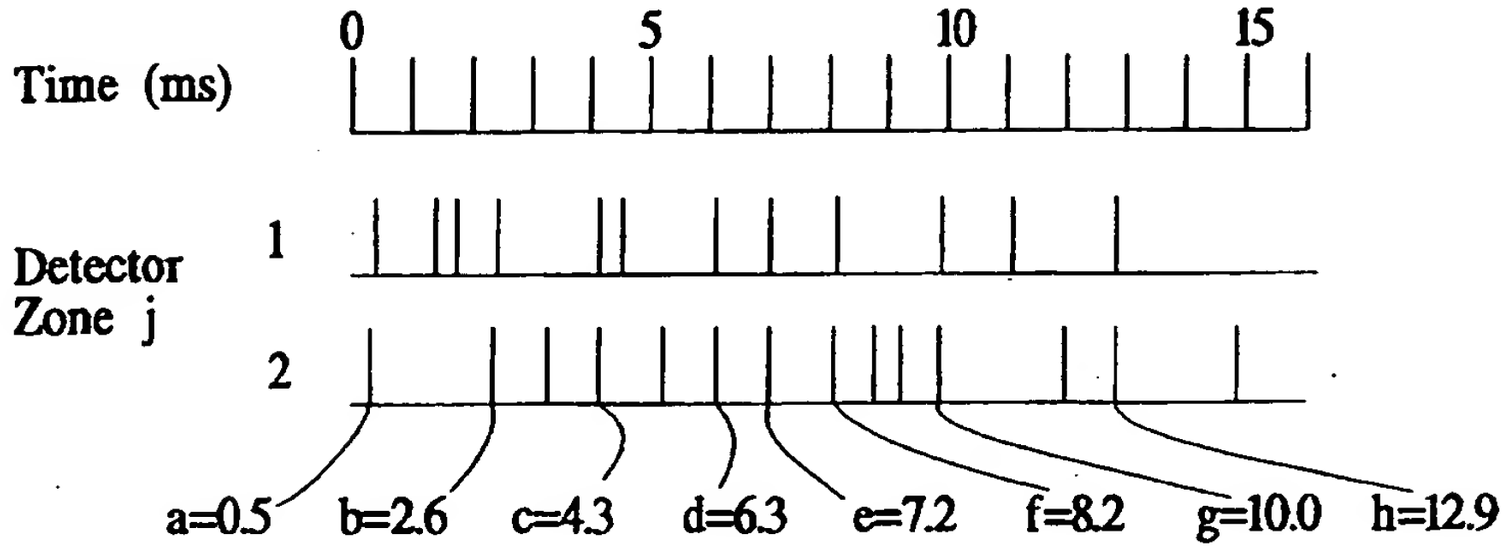

Null
BT gene
BT
400
200
0
-200
-400
-600
Time (msec)
2.6
2.4
2.2
2.0
1.8
1.6
1.4
Cross-correlation (au)

FIG. 5

FIG. 6A



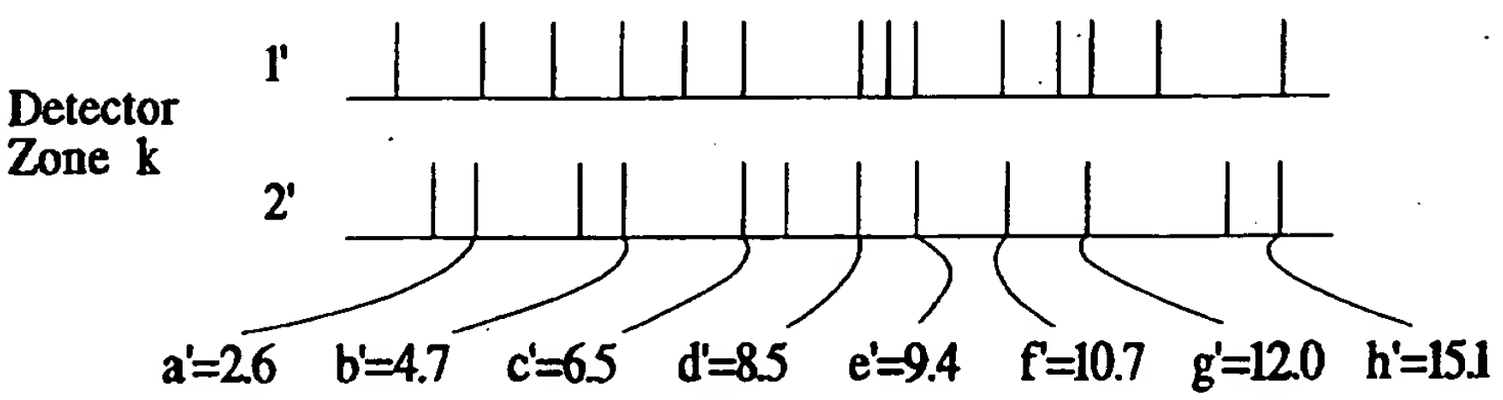


FIG. 6B

Δt_{jk}	a'	b'	c'	d'	e'	f'	g'	h'
a	2.1	4.2	6.0					
b		2.1	3.9	5.9				
c			2.2	4.2	5.1	6.4		
d			0.3	2.2	3.1	4.4	5.7	
e				1.3	2.2	3.5	4.8	
f				0.5	1.2	2.5	3.8	6.9
g						0.7	2.0	5.1
h								2.2

FIG. 6C

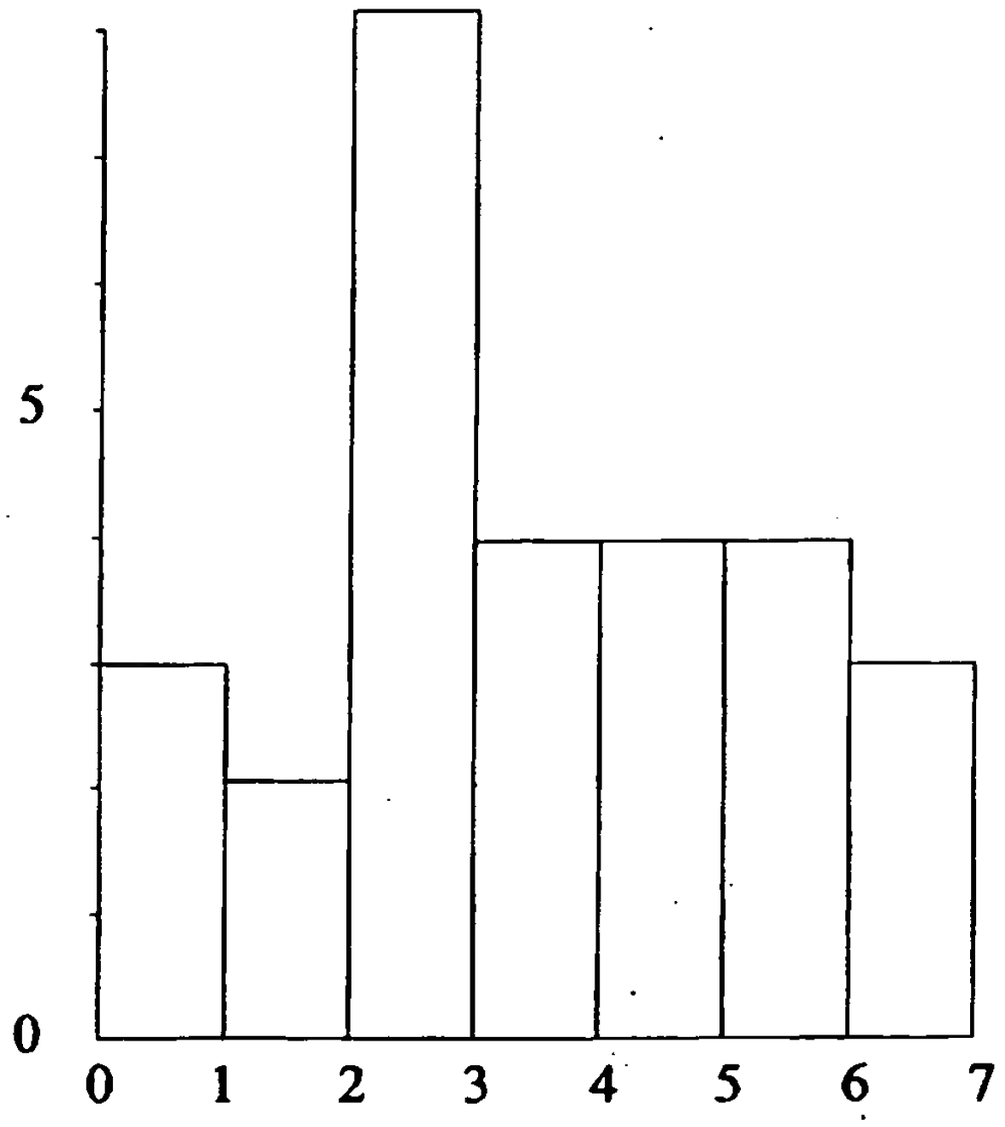
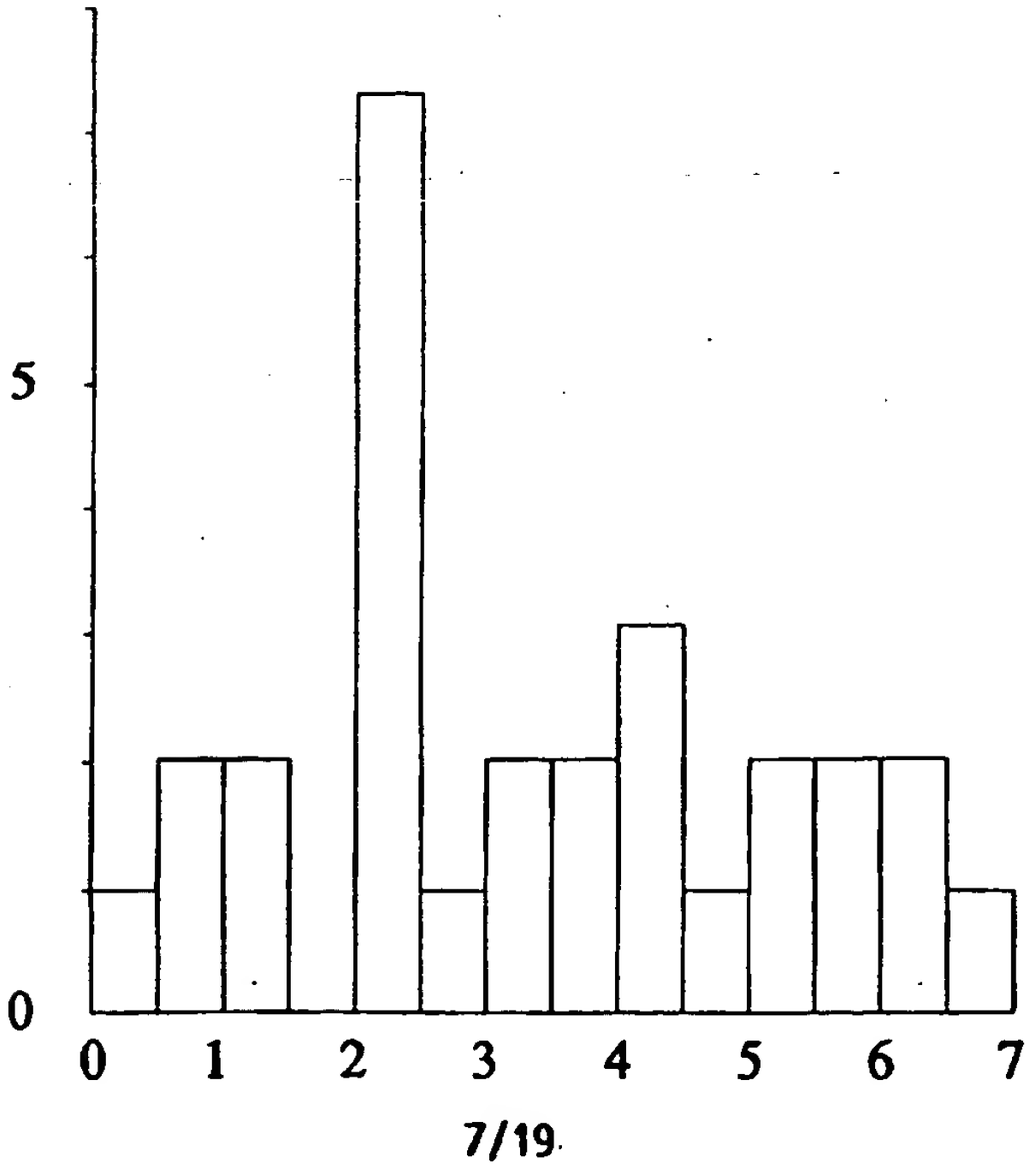


FIG. 6D



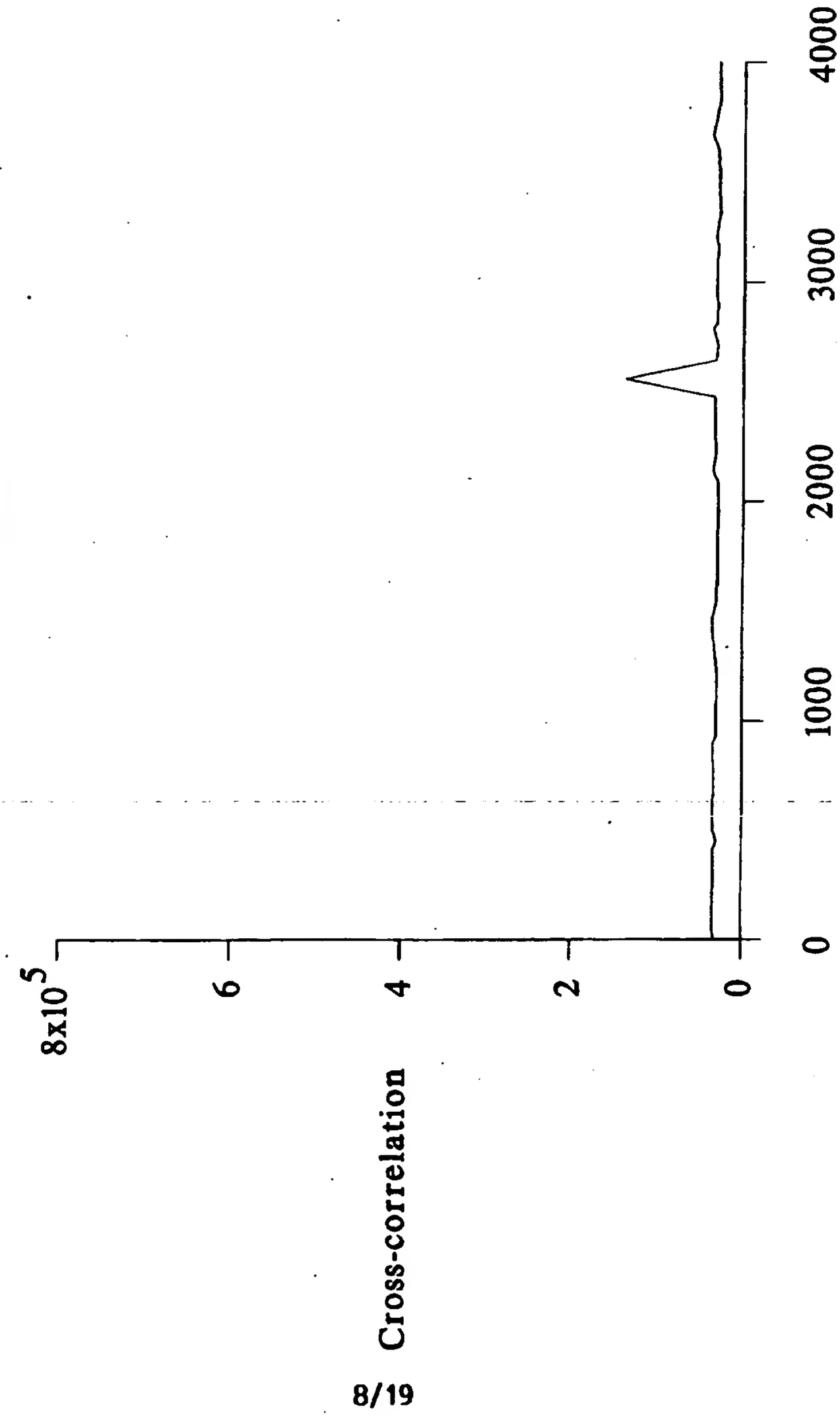

4000
3000
2000
1000
0
Time (msec)
8x10^5
6
4
2
0
Cross-correlation

FIG. 7

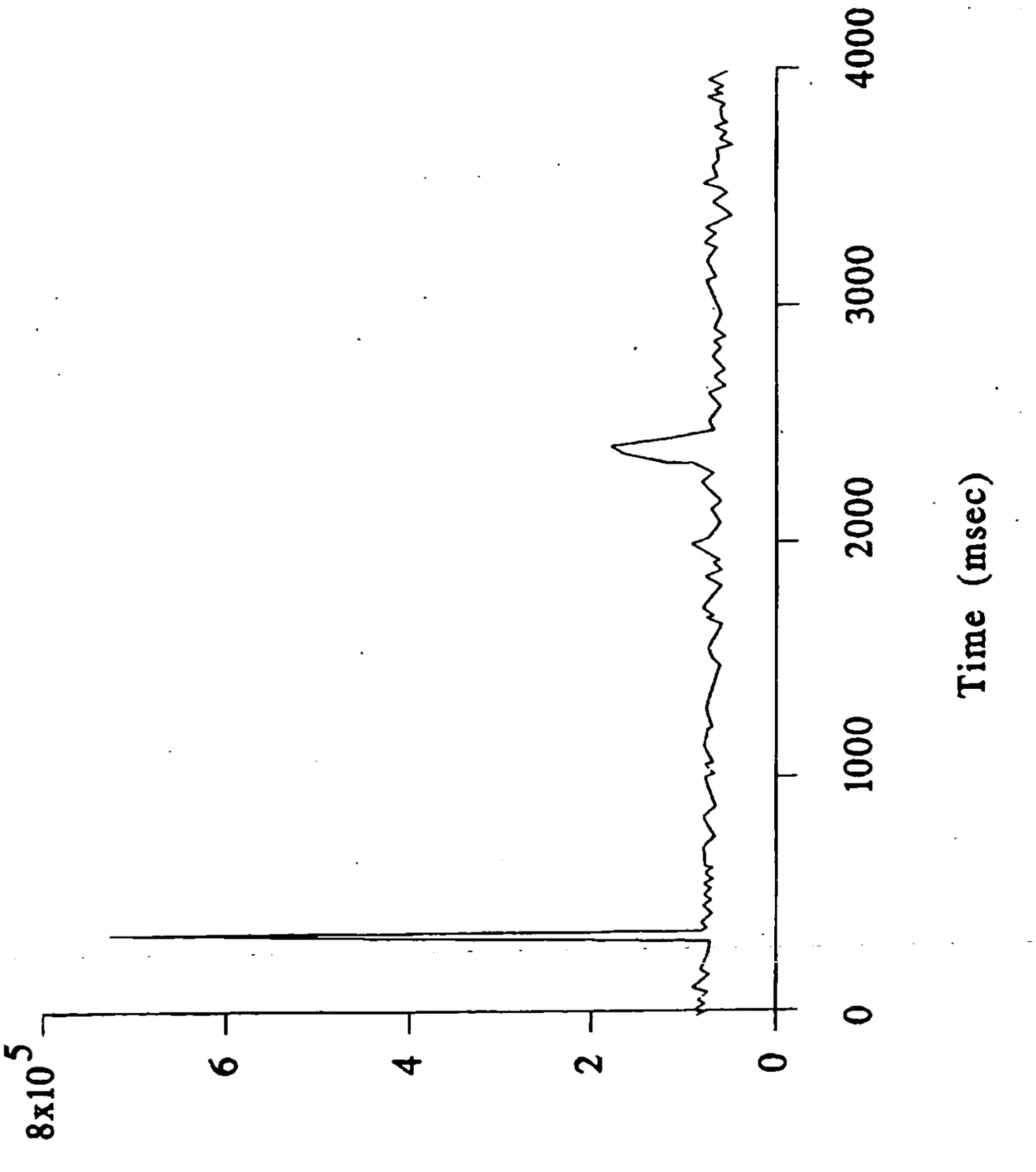
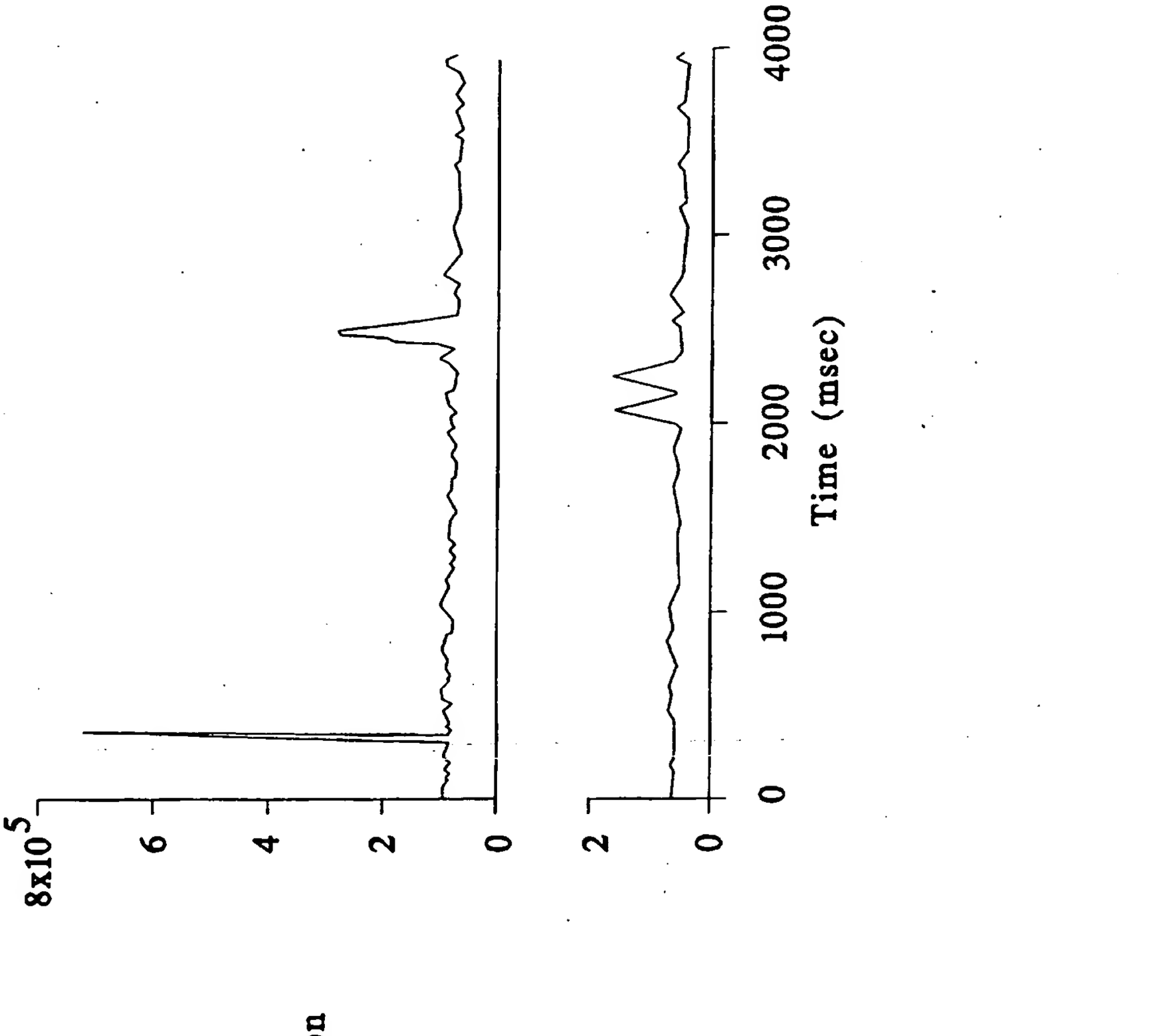

Cross-Correlation
8x10^5
6
4
2
0
0
1000
2000
3000
4000
Time (msec)

FIG. 8

FIG. 9


8x10^5
6
4
2
0
2
0
1000
2000
3000
4000
Time (msec)
Cross-Correlation

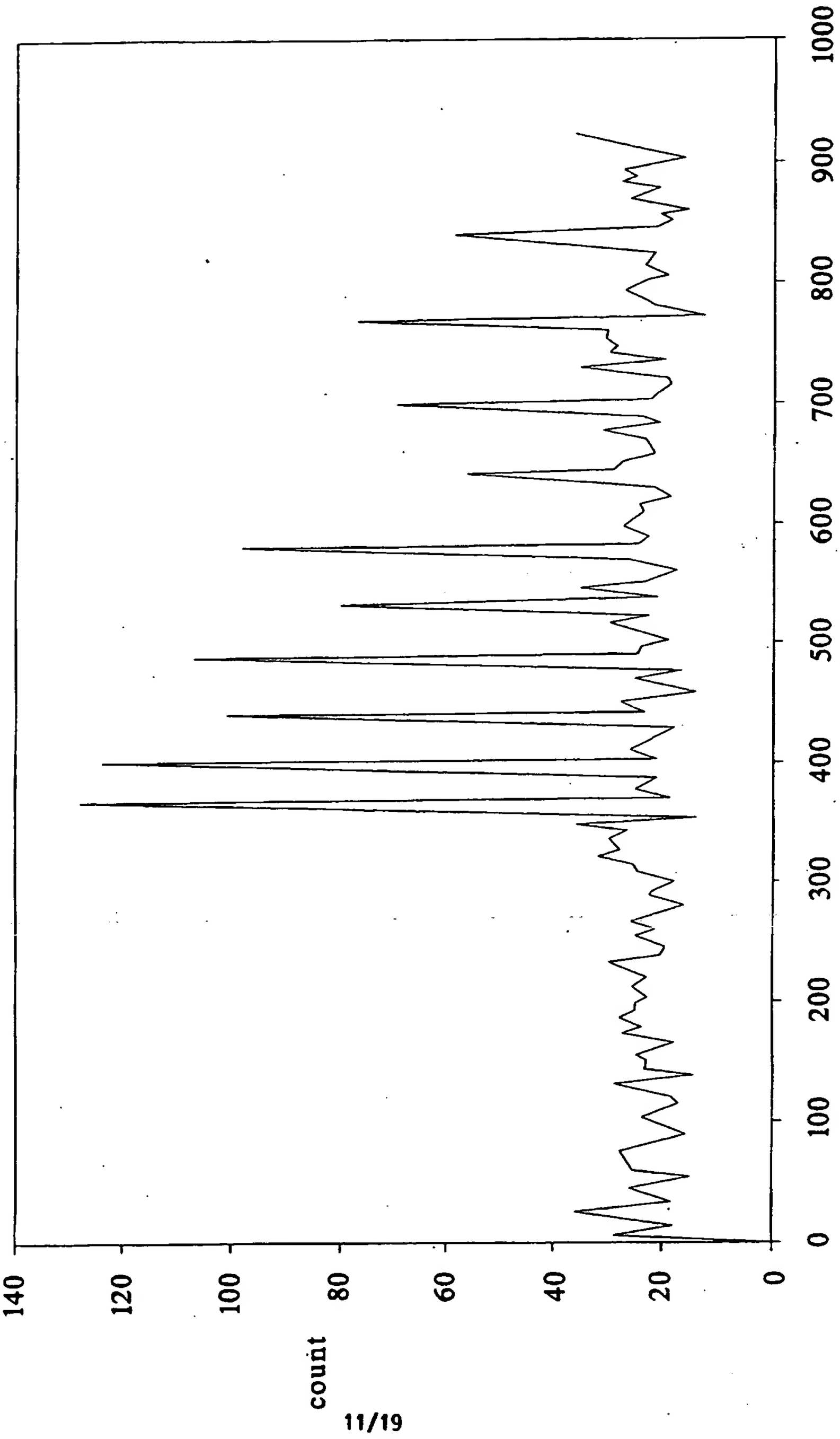

1000
900
800
700
600
500
400
300
200
100
0
msec
0
20
40
60
80
100
120
140
count

FIG. 10A

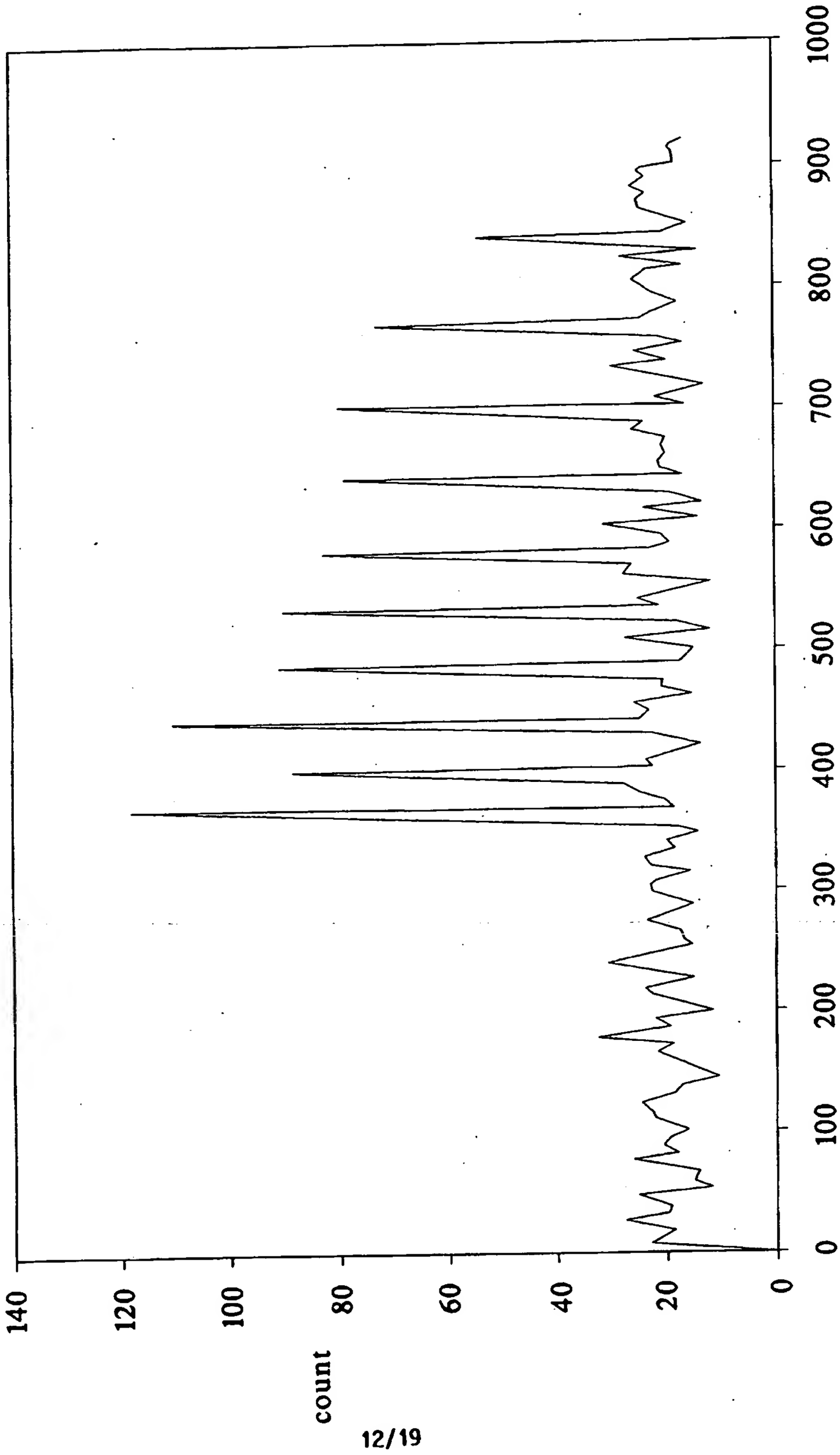

count
0
20
40
60
80
100
120
140
0
100
200
300
400
500
600
700
800
900
1000
msec

FIG. 10B

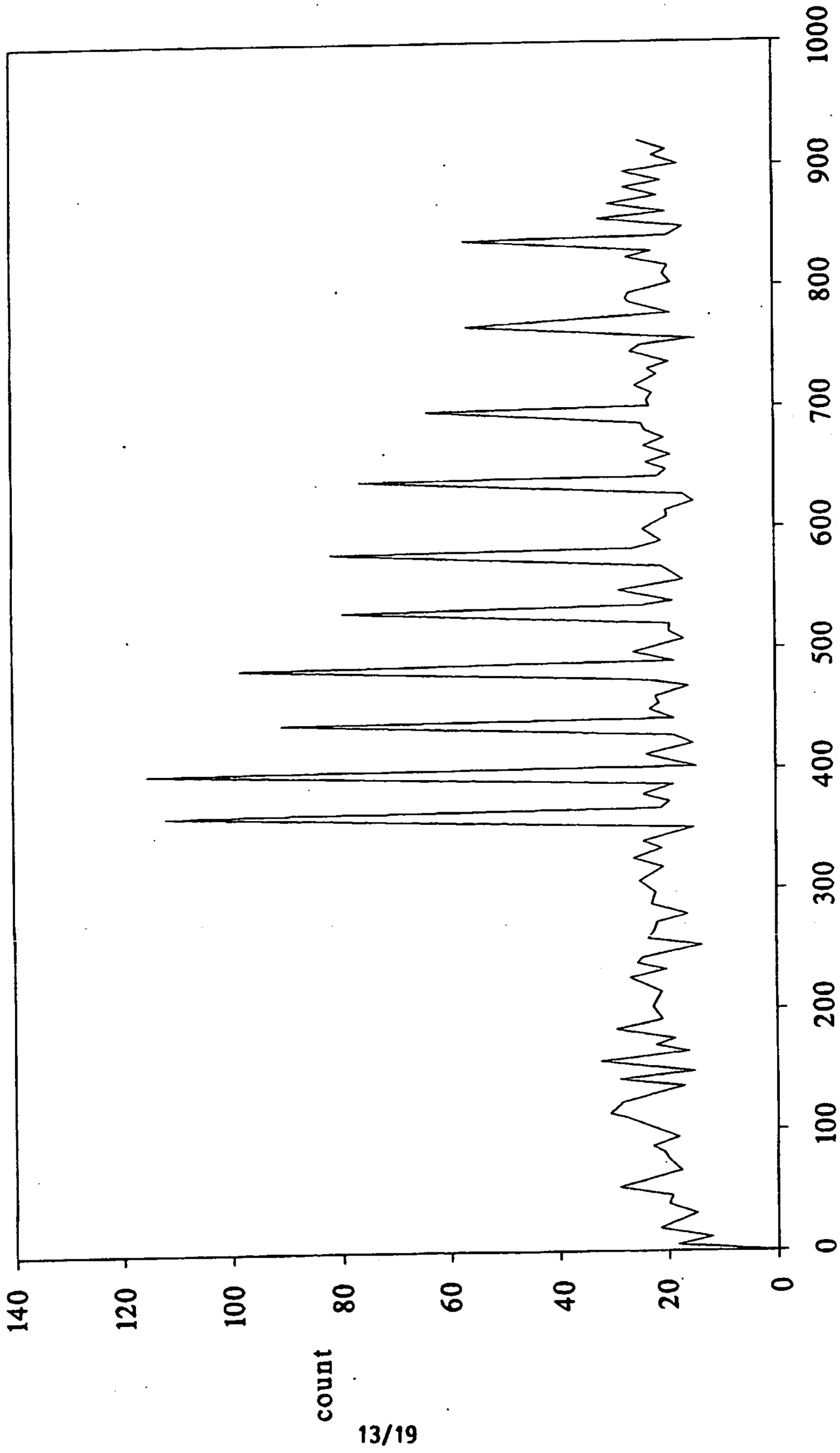

1000
900
800
700
600
500
400
300
200
100
0
msec
140
120
100
80
60
40
20
0
count

FIG. 10C

FIG. 11

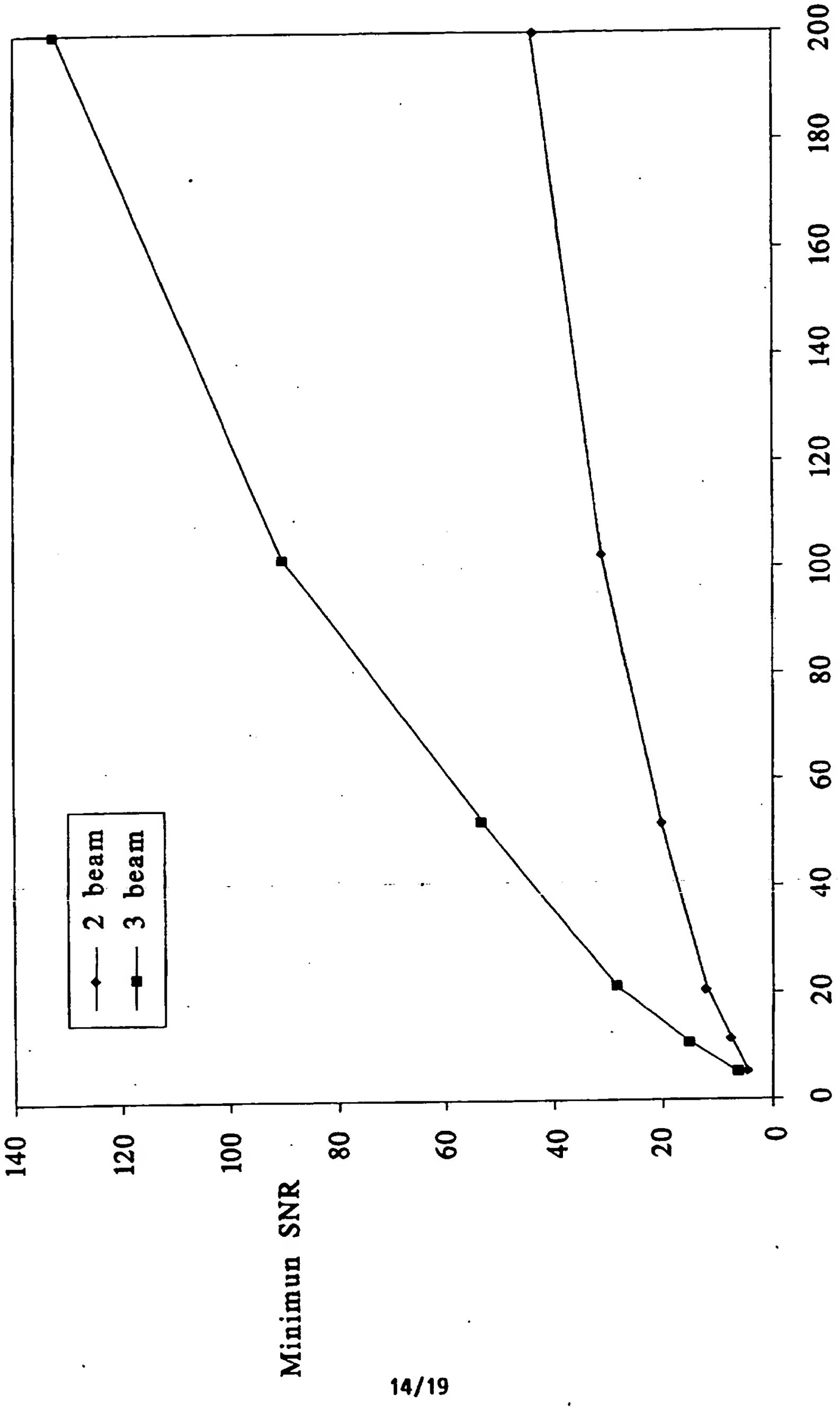
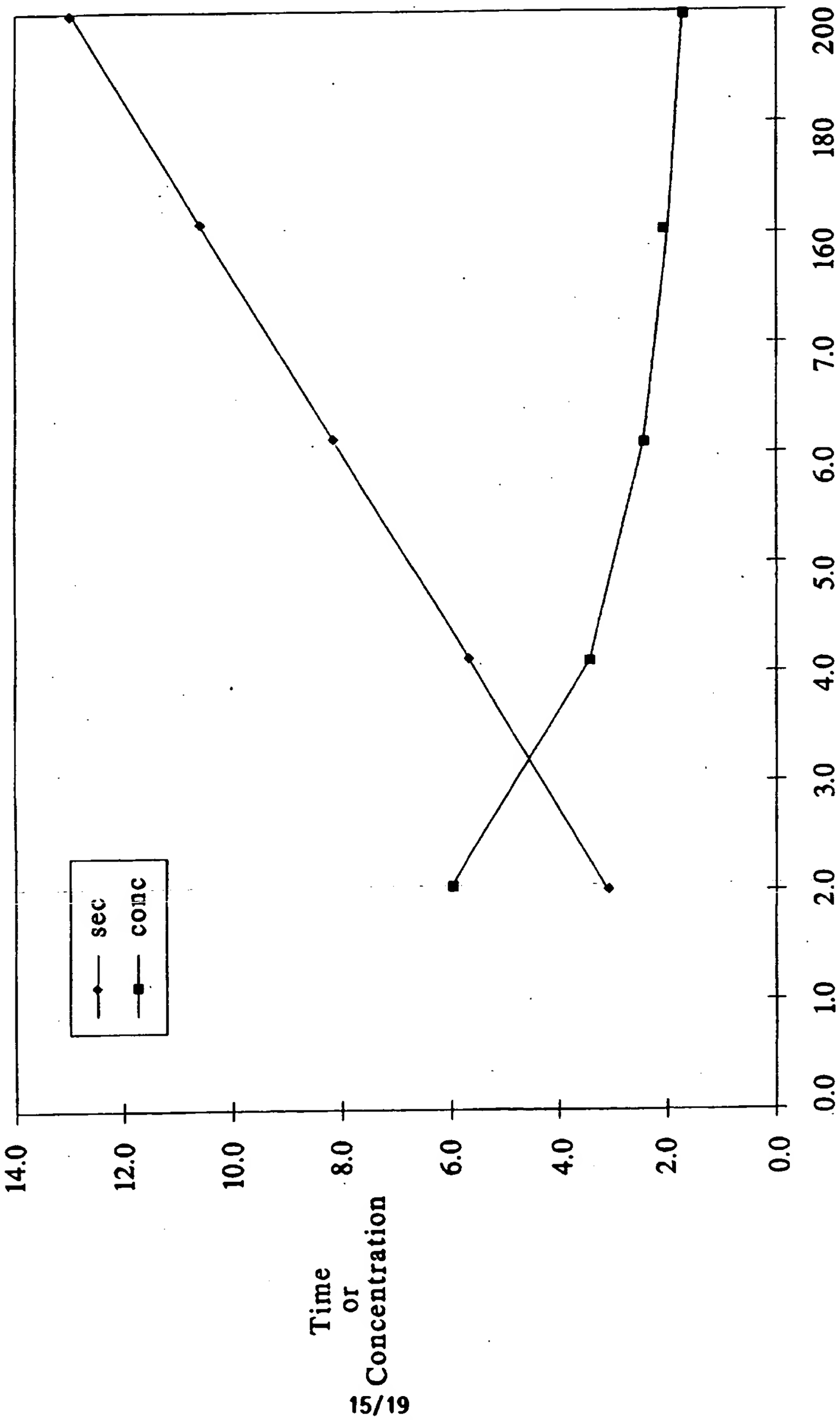

2 beam
3 beam
Minimun SNR
140
120
100
80
60
40
20
0
Genome Count
0
20
40
60
80
100
120
140
160
180
200

FIG. 12


sec
conc
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Time
or
Concentration
2.0
3.0
4.0
5.0
6.0
7.0
0.0
1.0
Detector Height, um
200
180
160

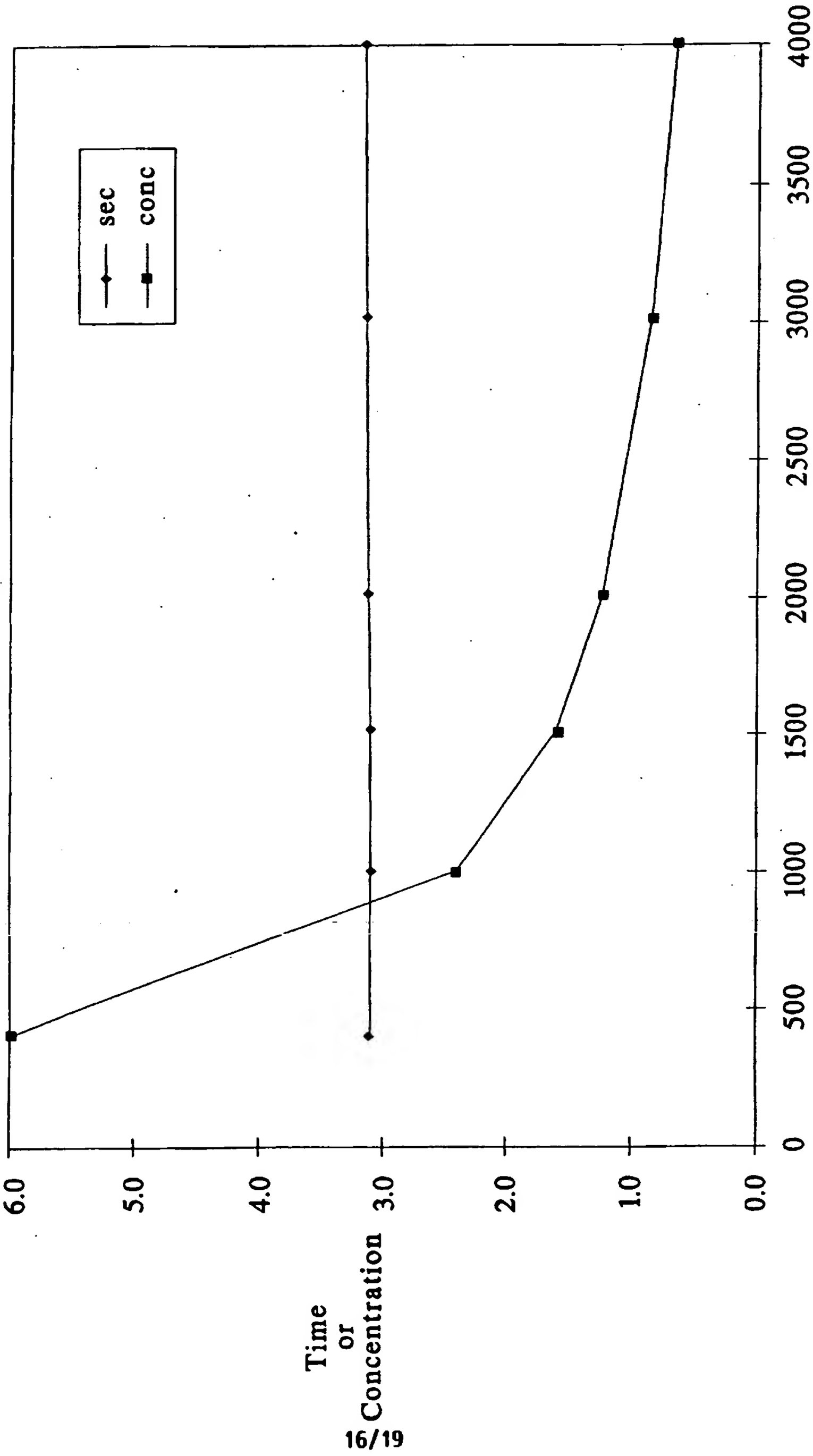

sec
conc
0
500
1000
1500
2000
2500
3000
3500
4000
Cross-Section Area, square microns
0.0
1.0
2.0
3.0
4.0
5.0
6.0
Time
or
Concentration

FIG. 13

FIG. 14

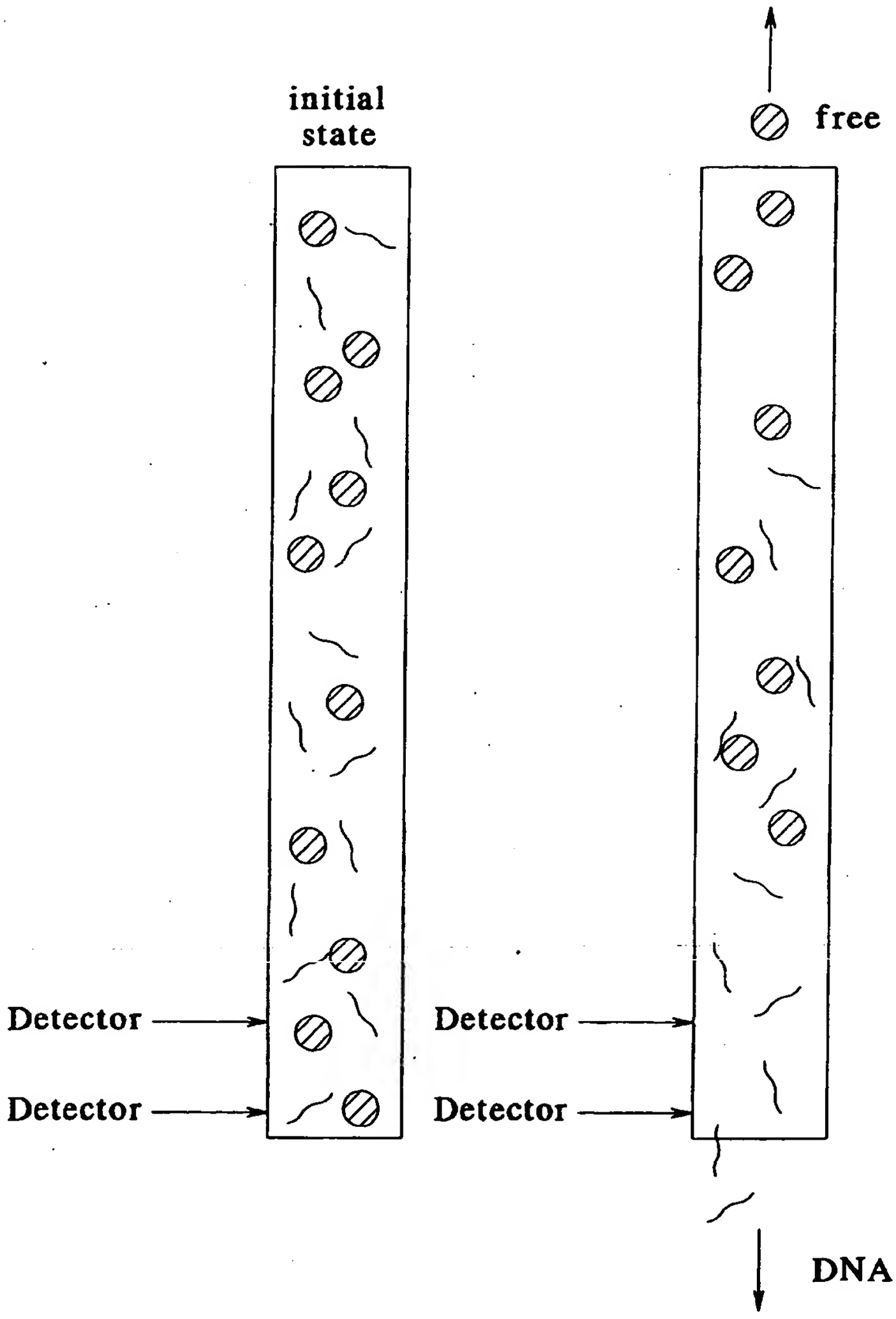

initial state
free probe
Detector
Detector
Detector
Detector
DNA

FIG. 15

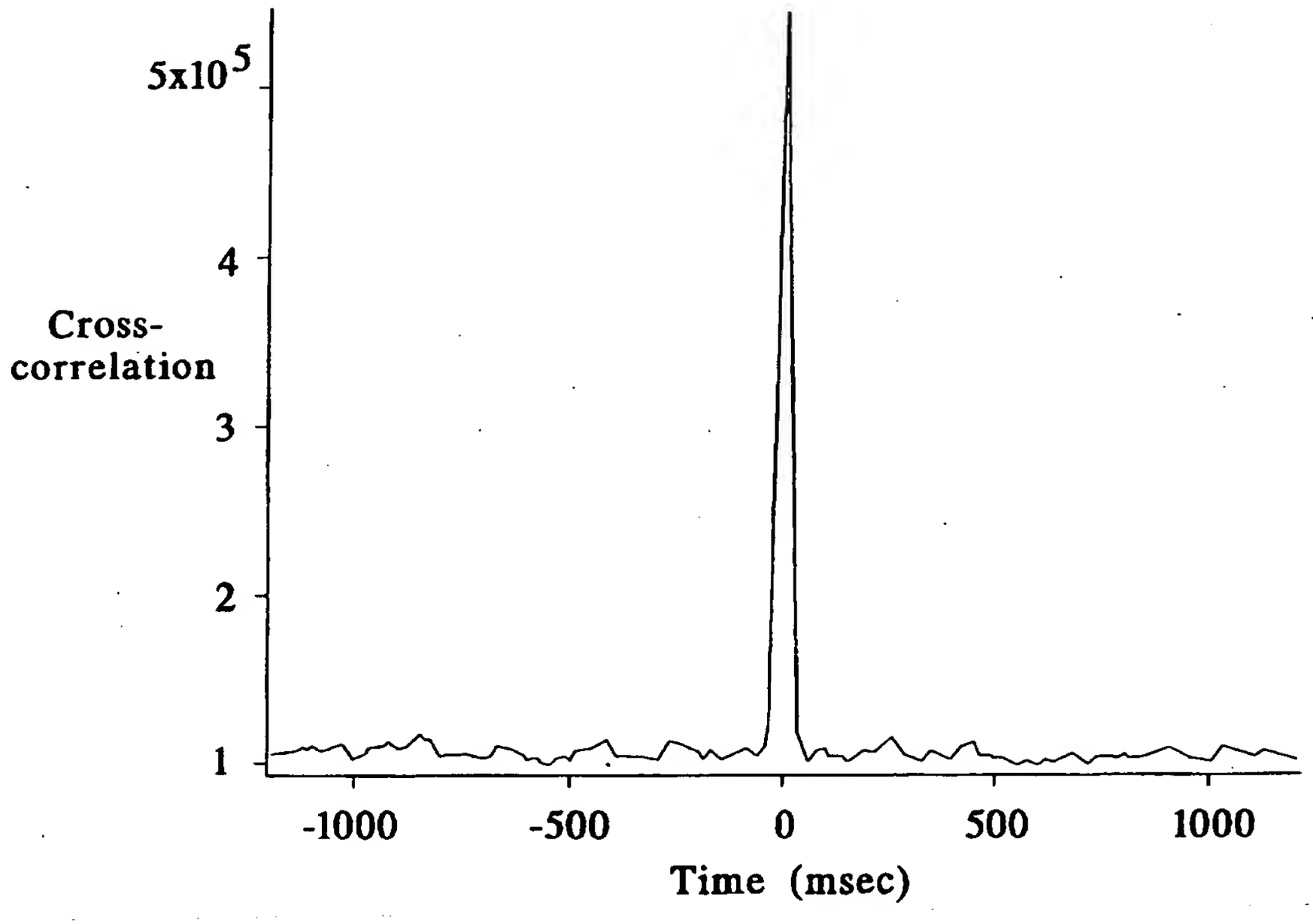


FIG. 16A

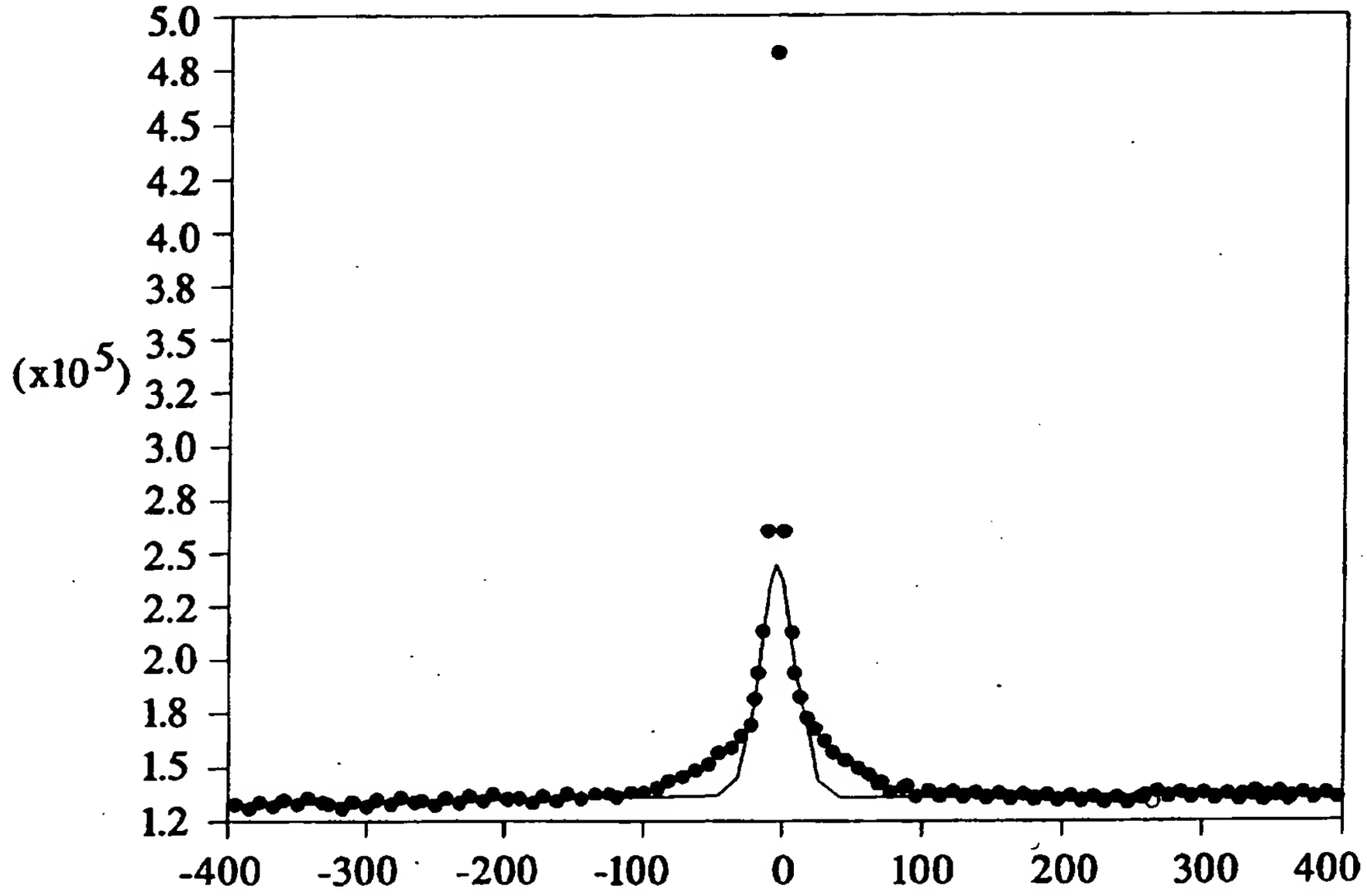


FIG. 16B